

# **The Role of Murine 11 $\beta$ -Hydroxysteroid Dehydrogenase Type 1 (11 $\beta$ HSD1) in The Metabolism of 7-Oxysterols**

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## **Declaration**

I hereby declare that this thesis was written by me and that the data published in this thesis are the result of my own work, performed at The University of Edinburgh under the supervision of Drs Ruth Andrew and Patrick WF Hadoke, and Professor Brian R Walker.

The work presented in chapter 3, with HEK293 cells transfected with human 11 $\beta$ HSD1 and H6PDH, was performed by Miss Rachel S. Dakin as part of an MSc Dissertation, and a collaborative work during the course of this PhD project.

The rest of the work in this thesis has not previously been submitted for any other degree or qualification.

Tijana Mitić  
Edinburgh, 2009.

## Abstract

7-Oxysterols constitute the major component (40%) of oxidized low-density lipoprotein (oxLDL). They arise in the body via auto-oxidation of cholesterol and are known to induce endothelial dysfunction, oxidative stress and apoptosis in the vascular wall, prior to development of atherosclerosis. A novel pathway has been described for hepatic inter-conversion of 7-ketocholesterol (7-KC) and 7 $\beta$ -hydroxycholesterol (7 $\beta$ OHC) by the enzyme 11 $\beta$ -hydroxysteroid dehydrogenase type-1 (11 $\beta$ HSD1), better known for metabolizing glucocorticoids. Inhibition of 11 $\beta$ HSD1 is atheroprotective and the potential underlying mechanism for this may involve altered metabolism and actions of glucocorticoids. However, alterations in the metabolism of 7-oxysterols may also play an important role in this atheroprotective effect.

The work described here addresses the hypotheses that (i) 7-oxysterols are substrates for murine 11 $\beta$ HSD1; (ii) inhibition of 11 $\beta$ HSD1 may abolish cellular metabolism of 7-oxysterols; (iii) this route of metabolism may modulate the actions of 7-oxysterols and glucocorticoids on murine vascular physiology.

Murine 11 $\beta$ HSD1 inter-converted 7-oxysterols ( $K_m=327.6\pm98\mu\text{M}$ ,  $V_{\text{max}}=0.01\pm0.001\text{pmol}/\mu\text{g}/\text{min}$ ) but the regulation of reaction direction is different from that for glucocorticoids. Predominant *dehydrogenation of 7 $\beta$ OHC to 7-KC* was quantified in several models (recombinant protein, cultured cells stably transfected with 11 $\beta$ HSD1), in which predominant *reduction of glucocorticoids* was measured. Furthermore, in murine hepatic microsomes, *dehydrogenation of 7 $\beta$ OHC* occurred exclusively. In aortic rings in culture, however, both reduction and dehydrogenation of 7-oxysterols were evident. 7-Oxysterols and glucocorticoid substrates competed for metabolism by 11 $\beta$ HSD1, with 7 $\beta$ OHC inhibiting dehydrogenation of glucocorticoids ( $K_i=908\pm53\text{nM}$ ).

The circulating concentrations of 7-oxysterols in the plasma of C57Bl6 and 11 $\beta$ HSD1<sup>-/-</sup> mice were in the  $\mu$ M range (0.02 – 0.13 $\mu$ M). The disruption of 11 $\beta$ HSD1 has resulted in increased ratios of 7-KC and 7 $\beta$ OHC over total plasma cholesterol levels (\* $p < 0.05$ ). This finding suggested that 11 $\beta$ HSD1 is involved in metabolizing and determining the plasma levels of 7-KC and 7 $\beta$ OHC.

To assess the consequences of these alterations for vascular function, studies were undertaken in aortic rings. Prolonged incubation with 7-oxysterols (20-25  $\mu$ M) showed a tendency to attenuate noradrenaline-mediated contractions of C57Bl6 aortae, but had no effect on contractions in response to 5-hydroxytryptamine or KCl. Similarly, endothelium-dependent and -independent relaxations of murine aortae were unaltered after exposure to 7-oxysterols.

Thus in the mouse, 11 $\beta$ HSD1 may influence the balance of circulating and cellular 7-oxysterols which may have consequential effects on glucocorticoid action. Although this work suggests that concentrations present in murine tissues are unlikely to cause vascular dysfunction, they may influence further cellular events as yet undescribed. Under pathological conditions where high concentrations of 7-oxysterols occur, 11 $\beta$ HSD1 may influence the extracellular-transport and delivery of 7-KC and 7 $\beta$ OHC to the plaque. This work therefore proposes that inhibition of metabolism of 7-oxysterols by 11 $\beta$ HSD1 inhibitors, may contribute to the atheroprotective effects of these drugs.



## **Awards and Presentations at Scientific Meetings**

Endocrine Society Travel Awards (2007 - 2009)

**2009** – ENDO Meeting Washington USA (£750);

**2009** – BES Meeting Harrogate UK (£500);

**2008** – ENDO Meeting San Francisco USA (£750);

**2007** – BES Meeting Birmingham UK (£500).

British Pharmacological Society Bain Fund (**2009**, £250)

**2009** – ENDO Meeting Washington USA;

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### **POSTER PRIZE**

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## List of Publications

### PAPERS

Mitić T, Walker R, Andrew R & Hadoke PWF. (2009) 7-Oxysterols Are Metabolised by 11 $\beta$ -Hydroxysteroid Dehydrogenase-1 (11 $\beta$ HSD1) within the Aortic Wall but Do Not Directly Influence Contractility. In preparation.

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### ABSTRACTS

1. Mitić T, Walker BR, Andrew R & Hadoke PWF. (2009) 7-Oxysterols are metabolized by 11 $\beta$ HSD1 within the aortic wall but do not directly influence contractility. Programme of the 91<sup>th</sup> Meeting of the Endocrine Society, P156.
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3. Mitić T, Webster SP, Walker BR, Hadoke PWF & Andrew R. (2008) US Endocrine Society Abstracts; Contrasting kinetics of 11 $\beta$ HSD1 metabolism of oxysterols and corticosteroids may contribute to predominant intracellular regeneration of glucocorticoids. Programme of the 90<sup>th</sup> Meeting of the Endocrine Society, P375.
4. Mitić T, McNae I, Webster SP, Wamil M, Walker BR, Hadoke PWF & Andrew R. (2007) Endocrine Abstracts, 11 $\beta$ -Hydroxysteroid dehydrogenase-1: key regulator in oxysterol metabolism? 13 P167.

5. Mitić, T, McNae I, Webster SP, Wamil M, Walker BR, Hadoke PWF & Andrew R, (2007) Scottish Medical Journal, 11 $\beta$ -Hydroxysteroid dehydrogenase-1: key regulator in oxysterol metabolism? 52(2): 51-55.
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“Twenty years from now, you’ll be more disappointed by the things you didn’t do than by the things you did. Explore. Dream. Discover.”

Mark Twain, 1879

To my Family, Slađa, Gaci, Nana and Bojan

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## List of Abbreviations

A	11-dehydrocorticosterone
ABC	ATP-binding cassette
ABCA1	ABC transporter A1
ABCG1	ABC transporter G1
ACAT	Acyl-CoA : cholesterol acyltransferase
ACh	Acetylcholine
ACE	Angiotensin converting enzyme
ACTH	Adrenocorticotrophic hormone
ADX	Adrenalectomy
AME	Apparent mineralocorticoid excess
ANOVA	Analysis of variance
ApoA1	Apo lipoprotein A-1
ApoB	Apolipoprotein B
ApoE	Apo lipoprotein E
AUC	Area under the curve
B	Corticosterone
BHT	Butylated hydroxy toluene
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
BSTFA	Bis(trimethyl)silyltrifluoroacetamide
CBX	Carbenoxolone
cDNA	Complementary deoxyribonucleic acid
C/EBP	CAAT/enhancer-binding protein
2:1	Chloroform:methanol (2:1, containing 50 µg/ml BHT)
C4	4-Cholestenone
CE	Cholesteryl ester
D7-Chol	Deuterium            labelled            cholesterol 25,26,26,26,27,27,27 -d7
CHCl <sub>3</sub>	Chloroform
CHD	Coronary heart disease

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CHO	Chinese hamster ovary
CNS	Central nervous system
CO <sub>2</sub>	Carbon dioxide
CPM	Counts per minute
CRB	Corticotrophin-binding protein
CTX	Cerebroterminus xanthomatosis
CVD	Cardiovascular disease
CYP	Cytochrome P450
D7-7 $\beta$ OHC	Deuterium labelled 7 $\beta$ -OHcholesterol-25,26,26,26,27,27,27d7
D7-7-KC	Deuterium labelled 7-Ketocholesterol-25,26,26,26,27,27,27d7
DEX	Dexamethasone
11-DHDEX	11-dehydrodexamethasone
DHEA	Dehydroepiandrosterone
DKO	Double knockout
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E	Cortisol
ER	Endoplasmic eeticulum
F	Cortisone
FBS	Foetal bovine serum
FRET	Fluorescence resonance energy transport
FXR	Farnesoid X (bile acid) receptor
GC	Glucocorticoid
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GC/MS	Gas chromatography/mass spectrometry
G6P	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase

H <sub>2</sub> O	Water
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
<sup>3</sup> H-A	Tritiated <sup>3</sup> [H] <sub>4</sub> -1,2,6,7 – 11-dehydro-corticosterone
<sup>3</sup> H-B	Tritiated <sup>3</sup> [H] <sub>4</sub> -1,2,6,7 – Corticosterone
HCl	Hydrochloric acid
HDL	High density lipoprotein
HMDS	Hexamethyldisilazane
HPA	Hypothalamic-pituitary-adrenal
HPLC	High performance liquid chromatography
HSD	Hydroxysteroid dehydrogenase
HSP	Heat shock protein
11βHSD1	11β-Hydroxysteroid dehydrogenase type 1
11βHSD2	11β-Hydroxysteroid dehydrogenase type 2
5HT	5-Hydroxytryptamine
7βOHC	7β-Hydroxycholesterol
7αOHC	7α-Hydroxycholesterol
19-OHC	19-Hydroxycholesterol
22-OHC	22-Hydroxycholesterol
25-OHC	25-Hydroxycholesterol
27-OHC	27-Hydroxycholesterol
ICAM-1	Intercellular cell adhesion molecule-1
IGF	Insulin Growth Factor
IL	Interleukin
iNOS	Inducible nitric oxide synthase
7-KC	7-Ketocholesterol
KPSS	Potassium physiological salt solution
LDL	Low density lipoprotein
LDLR	Low density lipoprotein Receptor
L-NAME	G-nitro-L-Arginine-Methyl Ester
LPS	Lipopolysaccharide
LXR	Liver X receptor, Oxysterol receptor

m11 $\beta$ HSD1	Mouse recombinant 11 $\beta$ HSD1 protein
M $\beta$ CD	Methyl- $\beta$ -cyclodextrin
MCP-1	Macrophage chemoattractant protein
MMP	Matrix metalloproteinase
MR	Mineralocorticoid receptor
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MO-TMS	Methoxyamine-trimethylsilimidazole
NaOH	Sodium hydroxide
NH	Nuclear hormone
NR	Nuclear receptor
NA	Noradrenaline
NAD <sup>+</sup> /NADH	Nicotinamide adenine dinucleotide (oxidized/reduced)
NADP <sup>+</sup> /NADPH	Nicotinamide adenine dinucleotide phosphate (oxidized/reduced)
NF $\kappa$ B	Nuclear factor $\kappa$ B
NO	Nitric oxide
NOS	Nitric oxide synthase
OFN	Oxygen free nitrogen
O <sub>2</sub>	Oxygen
O <sub>2</sub> <sup>-</sup>	Superoxide anion
OHC	Hydroxycholesterol
OxLDL	Oxidation modified low-density lipoprotein
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PDL	Poly-D-Lysine
PE	Phenylephrine
PEPCK	Phosphoenolpyruvate carboxykinase
PET	Position emission tomography



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PR	Percentage recovery, %
p/s	Penicillin/streptomycin
PSS	Physiological saline solution
PXR	Pregnane X receptor
RAR $\alpha$	Retinoic acid activates receptor $\alpha$
RCT	Reverse cholesterol transport
RLU	Relative light units
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RME	Relative error of means
RT	Room temperature
SCAD	Short chain alcohol dehydrogenase
SDR	Short-chain dehydrogenase/reductase
SEM	Standard error of the mean
Ser	Serine
SHP-1	Small heterodimer partner-1
siRNA	Short interfering RNA
SNP	Sodium nitroprusside
SR-A	Scavenger Receptor A
SR-BI	Scavenger Receptor BI
$t_{1/2}$	Half life
TC	Tissue and cell culture
TCMS	Trichloromethylsilazine
TMSI	Trimethylsilylimidazole
TNF $\alpha$	Tumour necrotic factor $\alpha$
Tyr	Tyrosine
Val	Valine
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VLDL	Very low-density lipoprotein
v/v	Volume to volume ratio
VSMC	Vascular smooth muscle cell

## **Chapter 1. Introduction**

## 1.1. Introduction

Dyslipidaemia is a component of the “Metabolic Syndrome” and is strongly associated with an increased risk of atherosclerosis, type 2 diabetes, obesity and fatty liver disease (Beaven and Tontonoz, 2006; Paterson *et al.*, 2004). In all of these conditions, excess cholesterol alters the plasma lipid profile, which further influences cholesterol storage and clearance by the body. Excess steroid hormones (glucocorticoids, GCs) can also contribute to the development of these diseases and has been linked with increased cardiovascular events (Hadoke *et al.*, 2009; Walker, 2007). Although many groups have suggested a link between increased glucocorticoid concentrations in tissues, particularly adipose, and the metabolic syndrome, the contribution of these steroids is not fully understood (Andrew *et al.*, 1998; Livingstone *et al.*, 2000; Morton *et al.*, 2005; Tomlinson *et al.*, 2004b). Tissue-specific increases in their levels are believed to be due to the actions of an enzyme, which reactivates intracellular glucocorticoid levels, 11 $\beta$ -hydroxysteroid dehydrogenase type 1 (11 $\beta$ HSD1). Indeed pharmacological inhibition of this enzyme is beneficial in reducing cardiovascular risk factors (lowering weight and glucose levels) and also in ameliorating cardiovascular disease (reduced atheroma) (Hermanowski-Vosatka *et al.*, 2005). This enzyme can metabolise substrates other than glucocorticoids, and this thesis will explore its role in regulating the concentrations and actions of 7-oxysterols.

## 1.2. 11 $\beta$ -Hydroxysteroid Dehydrogenases

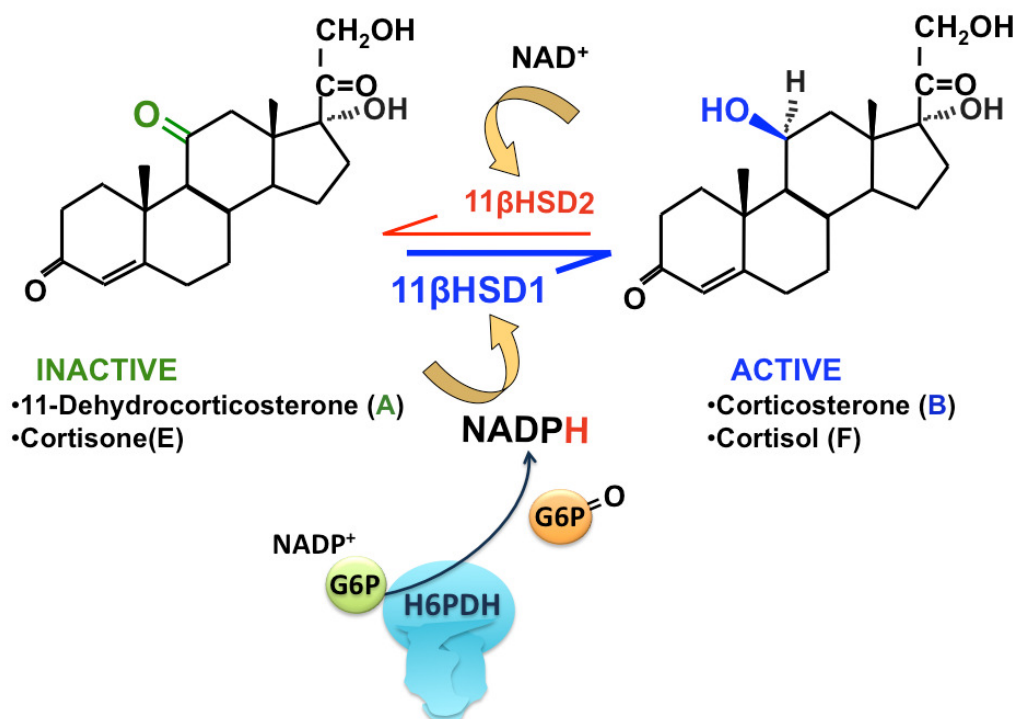
Various short chain alcohol dehydrogenases (SCADs) have been identified, constituting a large evolutionarily-conserved family of enzymes (Oppermann *et al.*, 2003). This class includes mammalian enzymes (expressed in placenta, liver and other tissues) such as 15-hydroxyprostaglandin, 17 $\beta$ -hydroxysteroid and 11 $\beta$ -hydroxysteroid dehydrogenases. Only about 20% homology exists between the molecular sequences of various dehydrogenases. Alignment of different SCAD enzymes reveals similarities between particular regions that are due to common

sequences which are known to be of special functional/structural importance (Filling *et al.*, 2002). Most known SCAD enzymes are NADH or NADPH-dependent oxidoreductases with distinct patterns of expression in tissues. Their generally accepted mechanism of catalysis is through conversion of the keto (=O) group on an individual molecule into a hydroxyl (-OH) group.

### **1.2.1. 11 $\beta$ -Hydroxysteroid Dehydrogenase Type 1 And Type 2: Biochemical And Physiological Characteristics**

11 $\beta$ -Hydroxysteroid dehydrogenases (11 $\beta$ HSDs) are microsomal enzymes that belong to the group of short chain alcohol dehydrogenases (Edwards *et al.*, 1988). Intracellular glucocorticoid levels are regulated by the actions of two isoforms of this enzyme: 11 $\beta$ -hydroxysteroid dehydrogenase type 1 (11 $\beta$ HSD1) and 11 $\beta$ -hydroxysteroid dehydrogenase type 2 (11 $\beta$ HSD2). These two isozymes of 11 $\beta$ HSD share about 20% sequence identities (Draper and Stewart, 2005).

11 $\beta$ HSD1 regenerates active glucocorticoid (cortisol (F) in human and corticosterone (B) in rodents) from inactive precursors (cortisone (E) in humans and 11-dehydrocorticosterone (A) in rodents; (Seckl and Walker, 2001)). The 11 $\beta$ HSD2 enzyme catalyzes the opposing dehydrogenation reaction (converting active glucocorticoids into their inactive forms; Figure 1.2 – 1) thus preventing inappropriate access of glucocorticoids to mineralocorticoid receptors (Stewart and Krozowski, 1999). These reactions occur in a tissue-specific manner with 11 $\beta$ HSD1 expressed predominantly in glucocorticoid target tissues (liver, lung, adipose tissue, gonads and brain; (Krozowski *et al.*, 1990)). The main 11 $\beta$ HSD2 expressing tissues are kidneys and placenta, but it is also expressed in gut, colon and blood vessels (Agarwal *et al.*, 1990). 11 $\beta$ HSD1 has a broader tissue expression than 11 $\beta$ HSD2, and its expression and activity are regulated by numerous complex mechanisms, including regulation by glucocorticoids themselves (Lanz *et al.*, 2001, Alikhani-Koopaei *et al.*, 2004, Heiniger, 2003).

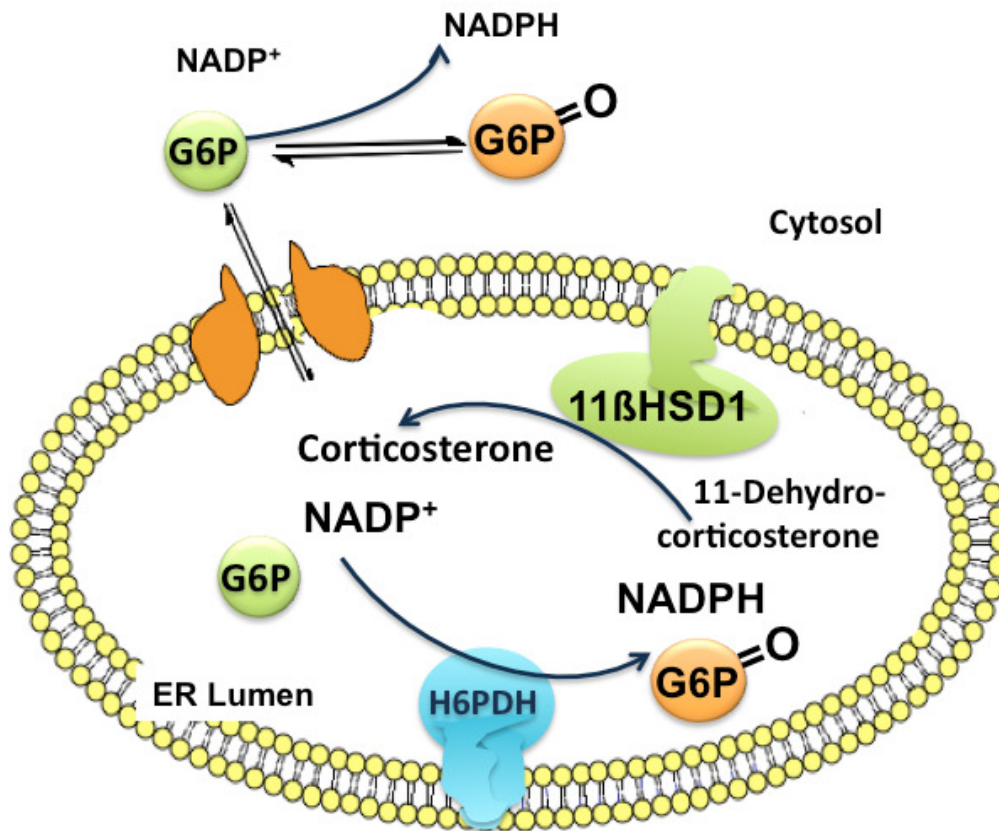


**Figure 1.2-1 Reactions catalyzed by 11 $\beta$ HSDs.**

Glucocorticoid inter-conversion by 11 $\beta$ -hydroxysteroid dehydrogenases (11 $\beta$ HSDs). In intact cells 11 $\beta$ HSD type 1 (11 $\beta$ HSD1) predominantly catalyzes reduction of 11-dehydrocorticosterone (A) to corticosterone (B); *activation reaction*. 11 $\beta$ HSD type 2 (11 $\beta$ HSD2) catalyzes the dehydrogenation of corticosterone (B) to 11-dehydrocorticosterone (A); *inactivation reaction*. The inactivation reaction catalyzed by 11 $\beta$ HSD2 utilizes NAD<sup>+</sup> as a cofactor, whereas the activation reaction catalyzed by 11 $\beta$ HSD1 utilizes NADPH. The NADPH is supplied by hexose-6-phosphate dehydrogenase (H6PDH), which converts glucose-6-phosphate (G6P) to 6-phospho-gluconolactone (6PG=O) thus generating NADPH for use by 11 $\beta$ HSD1.

11 $\beta$ HSD1 has a low affinity ( $K_m$  in the  $\mu M$  range) for both cortisol and corticosterone. It is an NADP(H)-dependent enzyme, that is a predominant reductase *in vivo*, catalyzing reduction of inactive A to active B (Jamieson *et al.*, 2000). *In vitro* in disrupted cell and tissue preparations 11 $\beta$ HSD1 acts as a bi-directional enzyme, catalyzing both reductase and dehydrogenase reactions. There are, however, a few studies reporting that 11 $\beta$ HSD1 may act as a dehydrogenase in intact cell preparations, depending both on cell type and differentiation status. In Leydig and neuronal cells *in vitro*, both reaction directions have been reported (Rajan *et al.*, 1996), whereas in human omental adipose stromal cells, 11 $\beta$ HSD1 switches from dehydrogenase to reductase upon cell differentiation into adipocytes (Bujalska *et al.*, 2002; Gao *et al.*, 1997). The directionality of this enzyme in the cell is influenced by cofactor availability. Reductase activity is driven by the generation of NADPH by an associate enzyme, hexose-6-phosphate dehydrogenase (H6PDH), within the endoplasmic reticulum (section 1.2.1.1). The N-terminal of 11 $\beta$ HSD1 anchors the enzyme to the membrane of the ER, while its active site faces into the lumen of the ER. This luminal orientation of 11 $\beta$ HSD1 is important for its reductase activity and is reliant upon microsomal supply of NADPH within the lumen (see Figure 1.2 – 2). In contrast, 11 $\beta$ HSD2 is a unidirectional NAD-dependent dehydrogenase with high affinity (nM range) for cortisone (Arnold *et al.*, 2003).

Although glucocorticoids are the principal substrates investigated in relation to 11 $\beta$ HSD enzymes, more recently cholesterol-derived sterols (7-oxysterols) (Hult *et al.*, 2004) and neurosteroids (e.g. 7-DHEA) (Nashev *et al.*, 2007), have been identified as substrates for 11 $\beta$ HSD1. This thesis investigates the role of 11 $\beta$ HSD1 in metabolism of these alternative substrates.

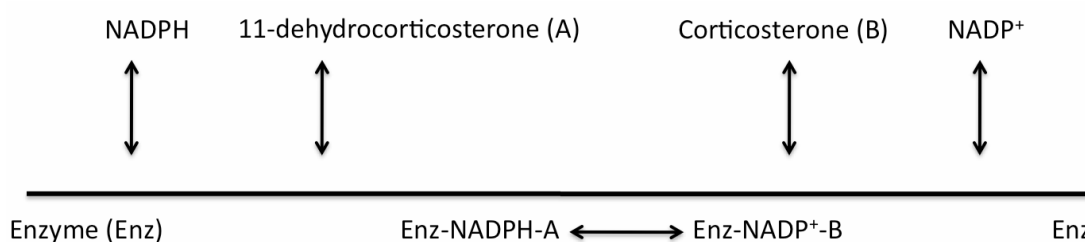


**Figure 1.2-2 Luminal orientation of 11 $\beta$ HSD1 in the endoplasmic reticulum.**

11 $\beta$ -Hydroxysteroid dehydrogenase type-1 (11 $\beta$ HSD1) is located on the luminal side of endoplasmic reticulum (ER) and its N-terminus is embedded into the membrane of the ER. The system comprising the glucose-6-phosphate (G6P) transporter (orange) and hexose-6-phosphate dehydrogenase (H6PDH) is crucial for transport of G6P to the H6PDH enzyme. G6P binds to the H6PDH to form 6-phospho-gluconolactone (G6P=O) resulting in generation of NADPH inside the lumen of the ER. The NADPH thus produced is utilized by 11 $\beta$ HSD1 for the reduction of 11-dehydrocorticosterone to corticosterone.

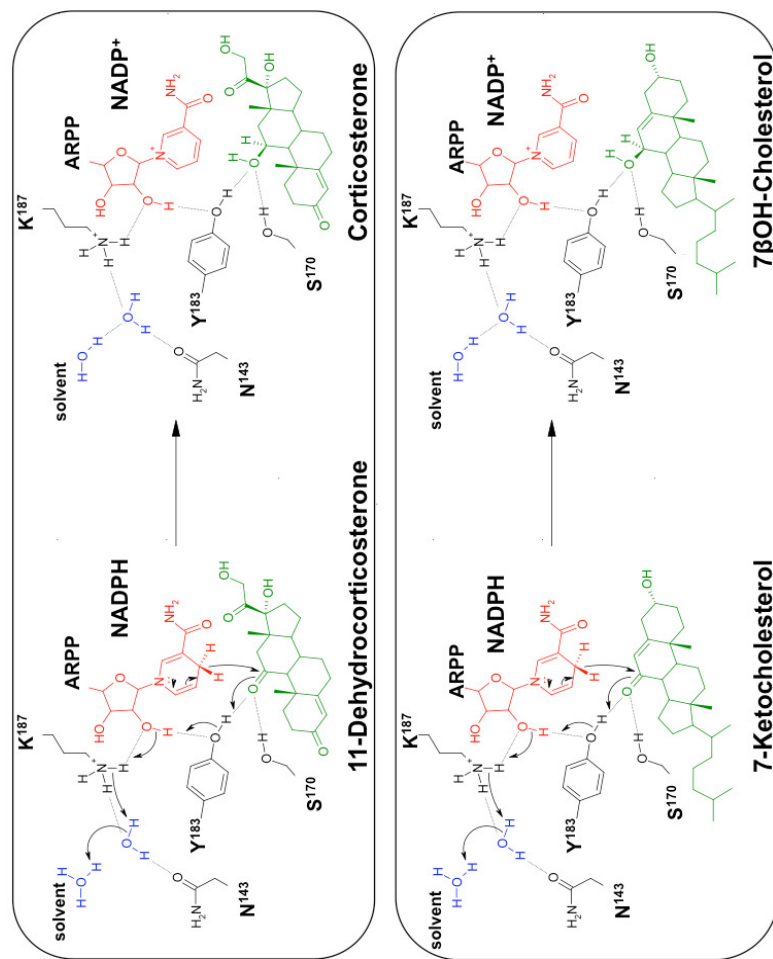
#### 1.2.1.1. Mechanism Of 11 $\beta$ HSD1 Reaction

The C-terminal part of 11 $\beta$ HSD1 contains the active site motif with a tetrad comprising the following amino acids: Asn, Ser, Tyr and Lys, catalytically essential for chemical reaction. Previously the triad of Ser – Tyr – Lys residues was considered, but more recent evidence by Filling *et al.*, have demonstrated a critical involvement of Asn residue in this process. The catalytic mechanism of 11 $\beta$ HSD1 in the active site requires interaction of the substrate with a resident cofactor (NADP<sup>+</sup>/NADPH) and with the tyrosine (Tyr<sup>183</sup>) and serine (Ser<sup>170</sup>) residues of the protein. This mechanism of 11 $\beta$ HSD1 reaction is also known as acid-base catalysis where substrate binding to the enzyme can be described according to a following mechanism of interaction (using Cleland-style notation for bi-substrate reaction, Castro *et al.*, (2007)):



Tyr residue is highly conserved in the family and is acting as a catalytic base in the deprotonation process. Ser stabilizes the substrate in the initial orientation and in the transition state. The protonated Lys forms hydrogen bonds with the ribose moiety of NADP<sup>+</sup> and lowers the pKa of Tyr-OH to facilitate proton transfer (Filling *et al.*, 2002). The presence of a conserved Asn residue is crucial in order to form a proton relay between the 2'OH of the nicotinamide ring and a conserved active site water molecule. The direct hydride transfer from the C4 position of NADP(H) to the carbon of the C11 ketone of the steroid (or C7 of 7-oxysterols) is facilitated by a general acid (see outlined mechanism in Figure 1.2 – 3).





**Figure 1.2 – 3 Chemical mechanism for 11βHSD1-catalysed reaction.**

The catalytic tetrad of 11βHSD1 consisting of asparagine (N<sup>143</sup>), serine (S<sup>170</sup>), tyrosine (Y<sup>183</sup>), and lysine (K<sup>187</sup>) is essential for the proton transfer between substrate (11-dehydrocorticosterone) and cofactor (NADPH). Tyr<sup>183</sup> is a conserved member of a catalytic tetrad proposed to function both as a general base and acid, depending on the reaction direction. The key steps of the proposed SDR chemical mechanism involve cofactor binding into the active site, followed by a substrate binding (Filling et al., 2002; Monder et al., 1991) (in this case dehydrocorticosterone). Catalysis begins with proton transfer from Tyr<sup>183</sup> hydroxyl to the substrate carbonyl, followed by hydride transfer to C11 (or C7) of the steroid (or oxysterol) and results in the formation of the reduced steroid corticosterone (or reduced oxysterol 7β-hydroxycholesterol). A similar mechanism occurs with also-keto reductase type of HSDs (Penning et al., 2003). ARPP, the adenosine ribose pyrophosphate moiety of NADPH.

### 1.2.1.2. **Hexose-6-Phosphate Dehydrogenase (H6PDH) Confers Predominant Reductase Activity Of 11 $\beta$ HSD1**

Hexose-6-phosphate dehydrogenase (H6PDH) utilizes glucose-6-phosphate as a substrate to provide the reduced cofactor NADPH (Stegeman and Klotz, 1979) for the 11 $\beta$ HSD1 reaction inside the endoplasmic reticulum (ER) (Hewitt *et al.*, 2005). The reductase activity of 11 $\beta$ HSD1 can be stimulated in rat liver microsomes by addition of the substrate for H6PDH (Banhegyi *et al.*, 2004). Perhaps most convincingly, mice with deletion in H6PDH (H6PDH<sup>-/-</sup>) cannot reduce 11-dehydrocorticosterone but show increased dehydrogenation of corticosterone (Lavery *et al.*, 2006). All of these studies indicate the reliance of 11 $\beta$ HSD1 on H6PDH-mediated generation of NADPH for its reductase function. Further expression analyses of H6PDH showed that this enzyme is present in a wide variety of tissues, but the greatest concentrations were in 11 $\beta$ HSD1 target tissues such as liver, kidney, and Leydig cells (although not as much was present in the brain (Gomez-Sanchez *et al.*, 2008)). This evidence also indicates tissue-specific regulation of glucocorticoid reactivation by 11 $\beta$ HSD1. It is now widely understood that an uncoupled redox-system exists between H6PDH and 11 $\beta$ HSD1 (Atanasov *et al.*, 2004; Piccirella *et al.*, 2006). Therefore, reduction of 11-dehydrocorticosterone is glucose-6-phosphate dependent, whereas oxidation of glucose-6-phosphate oxidation is dependent on the presence of 11-dehydrocorticosterone. There is recent *in vivo* evidence to show that the system comprising the glucose-6-phosphate transporter, H6PDH and 11 $\beta$ HSD1 is active in rat epididymal fat tissue, showing the importance of all three components for modulating local glucocorticoid activation (Marcolongo *et al.*, 2007).

It is assumed that perhaps dependence of 11 $\beta$ HSD1-metabolism on the generation of NADPH and co-operative action of H6PDH with 11 $\beta$ HSD1 is also important in the metabolism of 7-KC (as has been shown for 7-DHEA, a neurosteroid (Nashev *et al.*, 2007)). Therefore, an increase in 11 $\beta$ HSD1 activity by reduction of 7-KC is anticipated upon *in vitro* co-expression of H6PDH with 11 $\beta$ HSD1. However, this has not been conclusively demonstrated for 7-oxysterol substrates. This may be may be

relevant for understanding the physiological role of 11 $\beta$ HSD1 in metabolising alternative substrates.

### 1.2.2. 11 $\beta$ HSD1 Expression In The Vasculature

As mentioned previously, it is well recognized that exposure to chronic glucocorticoid excess leads to metabolic disorders such as: diabetes, insulin resistance, obesity, glucose intolerance, dyslipidaemia and hypertension (Walker, 2007). Considerable evidence suggests that GCs can directly influence vascular function and structure; actions which may contribute to development of cardiovascular disease. Both glucocorticoid (GR) and mineralocorticoid (MR) receptors are expressed in vascular tissue (Brem *et al.*, 1999) and corticosterone can be an agonist for both (and exerts rapid signalling effects) in VSMCs (Molnar *et al.*, 2008). The downstream effects of activation of these receptors is associated with cardiovascular risk factors (hypertension) and alteration in the vascular response to injury (Hadoke *et al.*, 2006). A recent study suggested that corticosterone can activate MR in the VSMC and trigger the phosphorylation of proteins key to cell proliferation and differentiation (Molnar *et al.*, 2008). In aortic rings *ex vivo*, corticosterone has repeatedly been shown to enhance agonist-mediated contraction (Brem *et al.*, 1997; Ullian, 1999; Ullian *et al.*, 1996) and inhibit endothelium-dependent relaxation (Brem *et al.*, 1997; Ullian, 1999). This is not, however, without controversy as previous studies have reported contradictory findings for alterations of vascular function after exposure to glucocorticoids (Christy *et al.*, 2003; Molnar *et al.*, 2008). The mechanism(s) involved remain unclear but recent work has demonstrated both the presence of GC response elements in the endothelial nitric oxide synthase (eNOS) promoter region and glucocorticoid-mediated inhibition of eNOS expression (Liu *et al.*, 2008).

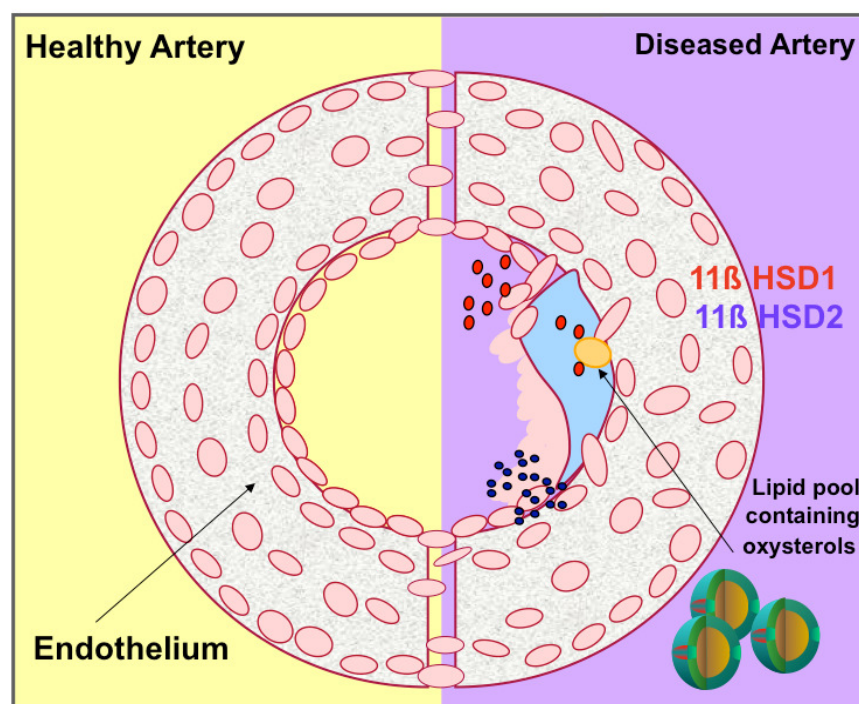
Studies of glucocorticoid metabolism in vascular tissue have demonstrated the presence of both 11 $\beta$ HSD1 and 11 $\beta$ HSD2 in vascular cells (Brem *et al.*, 1995; Hatakeyama *et al.*, 2001). Although controversy still exists concerning the exact localisation and direction of the activity of these enzymes, 11 $\beta$ HSD1 in the mouse aorta is expressed in VSMC (Walker *et al.*, 1991) and perhaps endothelial cells

(Christy, 2003; Hatakeyama *et al.*, 2001) whereas 11 $\beta$ HSD2 is localized to endothelial cells only (Cai *et al.*, 2001; Christy *et al.*, 2003; Morris *et al.*, 2003). An 11 $\beta$ HSD1-mediated increase in the local GC concentrations, leads to an activation of GR and MR (Seckl and Walker, 2001). This has significant implications for vascular homeostasis. Within the arterial wall 11 $\beta$ HSD1 influences GC-mediated changes in vascular contractility and structure (Hadoke *et al.*, 2006) and amplifies the angiostatic actions of GCs (Small *et al.*, 2005). 11 $\beta$ HSD2 on the other hand, prevents glucocorticoids from inhibiting endothelium-dependent vasodilation (Hadoke *et al.*, 2001). Pharmacological inhibition of 11 $\beta$ HSD1 dehydrogenase activity is associated with an enhanced response to vasoconstrictors, whereas inhibition of the reductase reaction produces attenuated constriction (Morris *et al.*, 2003).

### **1.2.3. Metabolism of Glucocorticoids By 11 $\beta$ HSD1 In The Vascular Wall: Influence On Atherogenesis**

During the development of atherosclerotic lesions the arterial wall undergoes considerable structural remodelling (Figure 1.2 – 4) leading to a complex environment involving monocyte accumulation, macrophage formation from differentiated monocytes and the uptake of oxidized lipids and cholesterol by macrophages to form foam cells. Increasing evidence suggests that 11 $\beta$ HSD1 activity may influence the processes underlying lesion development. For instance, the expression of 11 $\beta$ HSD1 in VSMC is up-regulated by pro-inflammatory cytokines, suggesting a mechanism for suppression of inflammation in the vessel by local generation of GCs (Cai *et al.*, 2001; Dover *et al.*, 2006). Furthermore, generation of GCs within macrophages may contribute to the regulation of inflammatory responses in these cells and inhibition of VSMC proliferation in the vessel wall (Chapman *et al.*, 2006). This suggests that local generation of GCs within the arterial wall may inhibit lipid accumulation and lesion formation. This has only been reported using exogenous GC administration (Asai *et al.*, 1993; Tauchi *et al.*, 2001).

It should also be noted, however, that beneficial effects of glucocorticoid generation on vascular health (such as the anti-inflammatory role of enhanced glucocorticoid generation by 11 $\beta$ HSD1), contrasts with recent demonstrations that 11 $\beta$ HSD1 inhibitors prevent atherosclerosis (Hadoke *et al.*, 2009; Hadoke *et al.*, 2008; Hermanowski-Vosatka *et al.*, 2005). One explanation is that the beneficial effects of the inhibitor are mediated through effects on alternative substrates, such as 7-oxysterols.



**Figure 1.2-3 Schematic diagram of the Arterial Wall - Healthy Artery versus an Atherosclerotic Artery.**

The composition of a healthy artery wall (in cross-section) is compared with that of an atherosclerotic artery. The latter illustrates the accumulation of a pool of lipid forming the core of an atherosclerotic plaque (adapted from (Freeman, 2005)). Chronic hyperlipidaemia/ atherosclerosis leads to damage of the endothelium by the action of oxidized lipids present in the plasma or from the lipid pool (containing oxysterols). Atheroprotective effects of 11 $\beta$ HSD1 inhibitors are associated with reduced cholesterol and oxidized lipid accumulation within the aortic plaque.

The role of glucocorticoids in the pathogenesis of metabolic and cardiovascular disease has prompted the suggestion that 11 $\beta$ HSD1 would be a valuable therapeutic target (Walker, 2007; Wamil and Seckl, 2007). Adding to the considerable body of evidence showing benefits of inhibition of 11 $\beta$ HSD1, development of these inhibitors may also ameliorate risk factors associated with cardiovascular diseases, such as type 2 diabetes (Hale and Wang, 2008). Certainly this is consistent with the demonstration that pharmacological inhibition of 11 $\beta$ HSD1 reduced cholesterol accumulation (and, by implication, lesion development) in an Apolipoprotein E knockout (ApoE<sup>-/-</sup>) mouse model of atherosclerosis (Hermanowski-Vosatka *et al.*, 2005). It is not clear, however, how inhibition of 11 $\beta$ HSD1 reduced atherosclerosis in these mice, although it was suggested that the reduction in aortic lipid accumulation could not be attributed solely to the mild reduction in circulating lipid concentrations. In a different study using low density lipoprotein (LDL) receptor knockout (LDLR<sup>-/-</sup>) mice, subcutaneous injection of carbenoxolone (an 11 $\beta$ HSD1 inhibitor, although non-selective) inhibited lesion development only in obese atherosclerotic animals (Nuotio-Antar *et al.*, 2007). Whether these effects of 11 $\beta$ HSD1 inhibition on lesion development are related to their inter-conversion of glucocorticoids, or is more to do with changes in metabolism of alternative substrates (such as 7-oxysterols), remains to be determined.

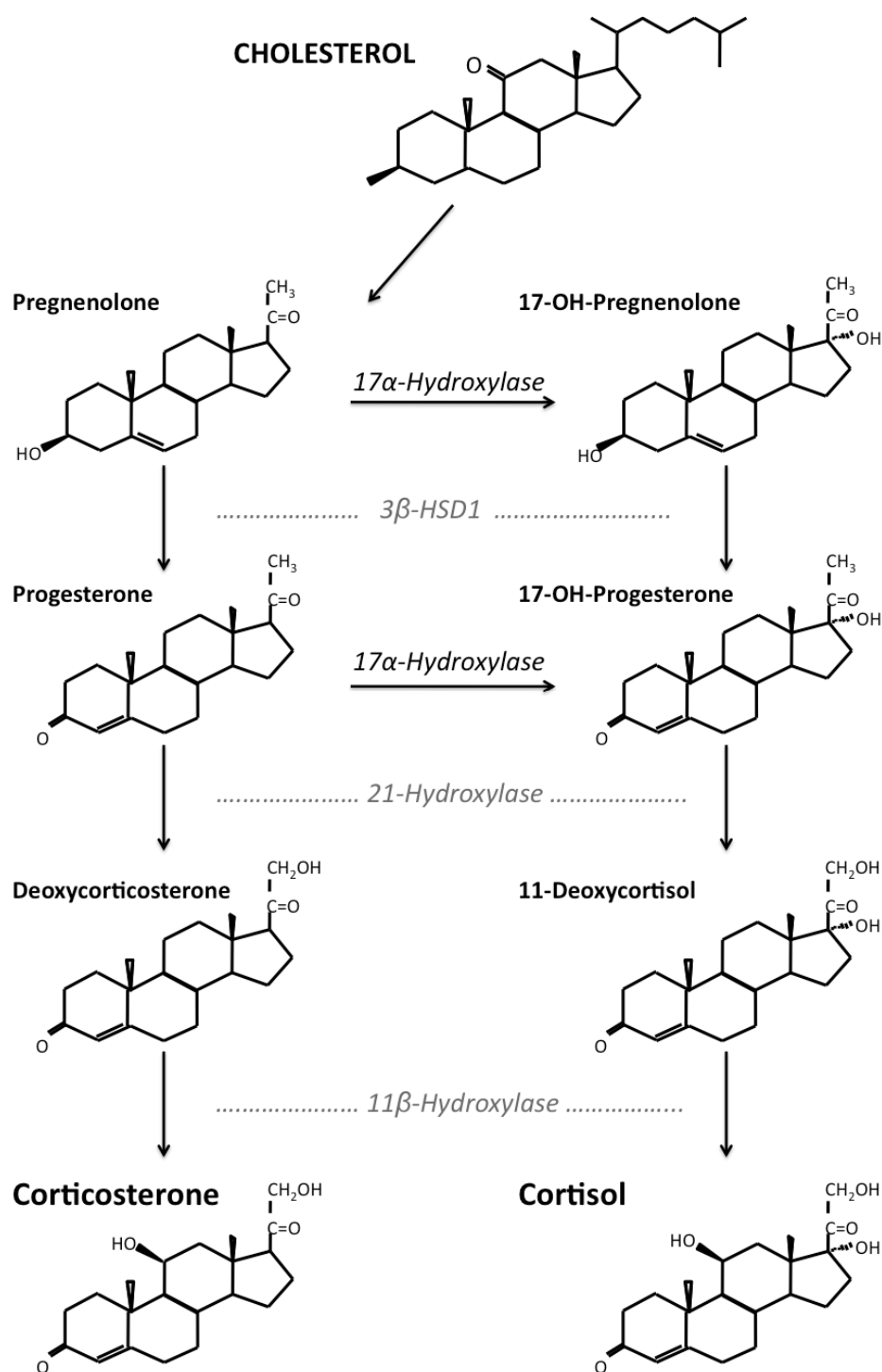
### 1.3. Oxysterols – Active Metabolites Of Cholesterol

The challenge of exploring cholesterol homeostasis was tackled by Goldstein and Brown following their description of xanthoma formation (abnormal lipid accumulation under the skin (Brown and Goldstein, 1974; Goldstein *et al.*, 1974)). Whilst cells in patients with this condition can produce low-density lipoprotein, it accumulates in the plasma and is, ultimately, stored under the skin (due to a dysfunction of the LDL receptor). The risk of a myocardial infarction at the age of six and the absence of constitutive activity of 3-hydroxy 3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase) in these patients, were later attributed to a disease known as homozygous familial hypercholesterolemia (FH) (for recent overview see Goldstein and Brown, (2009)). This discovery of dysfunctional LDL

receptor activity leading to vascular disease provided a novel ground for future research on atherogenesis and its treatment.

Cholesterol is the most important regulator of lipid organization in the cell membrane bilayer, is a vital precursor for bile acid synthesis and is the main precursor for synthesis both of glucocorticoids (Figure 1.3 – 1) and of oxysterols (Figure 1.3-2). Consequently, mammals have developed complex mechanisms for maintaining cholesterol concentrations within the body, with metabolism to oxysterols occurring predominantly in the liver. (Goldstein and Brown, 2009; Jessup and Kritharides, 2008; Maxfield and Tabas, 2005). Cholesterol is carried in the blood in different lipoprotein particles, and mainly in high-density lipoprotein (HDL; so-called “good” cholesterol) and LDL (“bad”-cholesterol). Regulation of cholesterol levels within cells is controlled by lipoproteins and reverse cholesterol transport (Ohashi *et al.*, 2005). The exogenous supply of cholesterol to cells and tissues is maintained by LDL, whereas HDL removes excess cholesterol from the cells. However, over-abundance of cholesterol, in association with elevation of LDL, can increase the risk of atherosclerosis (Libby, 2002a), in which excess cholesterol accumulates within cells such as macrophages within the vessel wall. Increased cholesterol can be toxic for cells and a number of strategies have evolved to export cholesterol or store it in its esterified form. HDL cholesterol exports cholesterol from the liver and foam cells back into the blood. This process is altered in the pathogenesis of atherosclerosis in which HDL levels are decreased, whereas LDL levels are elevated (Gaut and Heinecke, 2001).

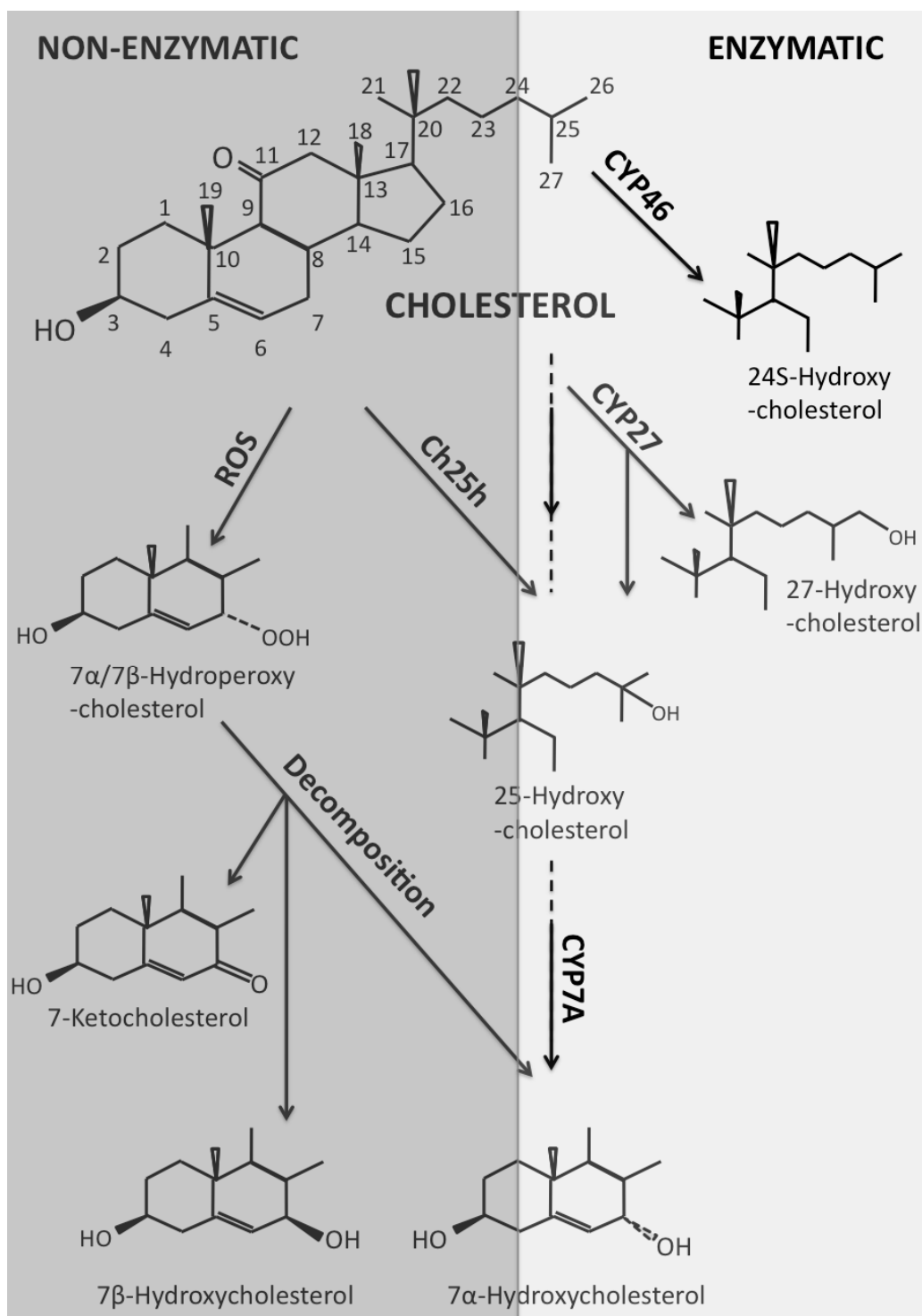
Reverse cholesterol transport is a pathway through which cholesterol is transported back to the liver from the peripheral organs (such as blood vessels). This represents a common excretion pathway, which also promotes cholesterol efflux and, thus, prevents atherosclerosis (Ohashi *et al.*, 2005). Clearance from tissue is mediated by



**Figure 1.3-1 Biosynthesis of glucocorticoids from cholesterol within the adrenal cortex.**

Biosynthesis of steroids starts from cholesterol and is catalyzed by many cytochrome P450 enzymes as labeled. The major active glucocorticoid in rodents is corticosterone since, unlike humans, rodents lack adrenal 17 $\alpha$ -hydroxylase.





**Figure 1.3-2 Biosynthesis of oxysterols from cholesterol.**

Biosynthesis of oxysterols starts from cholesterol and is catalyzed by many cytochrome P450 (CYP) enzymes, as labelled, and cholesterol 25-hydroxylase (Ch25h; which is a non-cytochrome P450 enzyme). Truncated structures of the oxysterols are shown, emphasizing the additional oxygenation. ROS, reactive oxygen species. Figure adapted from (Brown and Jessup, 2009).

members of the ATP-Binding Cassette (ABC)-transporter family: transporter A1 (ABCA1) and transporter G1 (ABCG1). Both of these transporters use the energy of ATP hydrolysis to facilitate cholesterol efflux from macrophages into Apolipoprotein AI and/or HDL (Baldan *et al.*, 2008). Much current research to treat atherosclerosis focuses on stimulating cholesterol efflux pathways from macrophages to HDL, with several mechanisms already described, including: ABC-mediated efflux; efflux through ApoE secretion; and SR-BI-mediated efflux (Tall *et al.*, 2008). Although it is not certain, it is also believed that these mechanisms provide a mechanism for clearing 7-oxysterols (and other hydroxy cholesterol, such as 27-hydroxycholesterol, 27-OHC) from the body.

### 1.3.1. Formation And Biological Sources Of Oxysterols

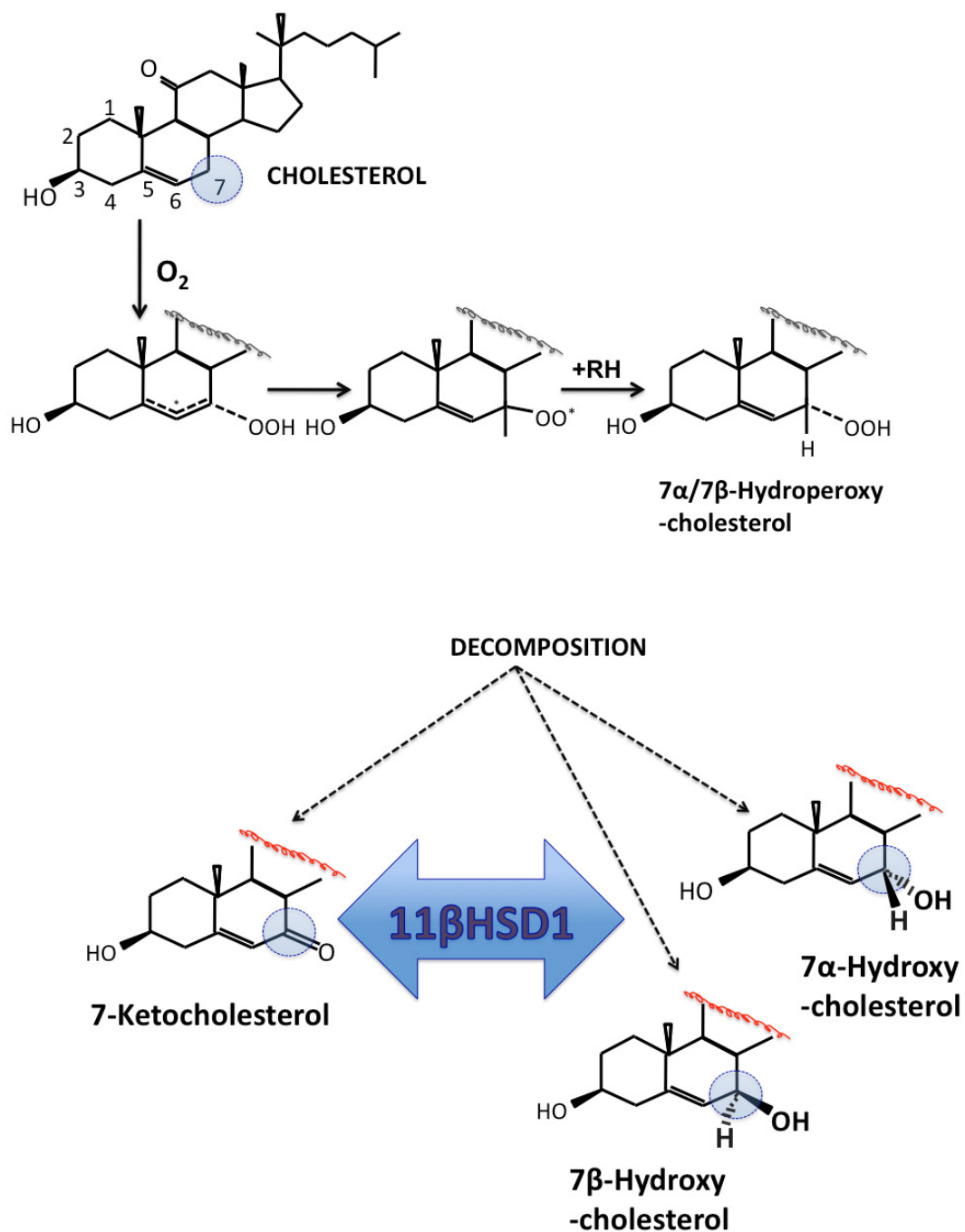
Circulating LDL particles contain lipids other than cholesterol: for example, oxysterols form their major (60%) component. Oxysterols are oxygenated derivatives of cholesterol or by-products of the cholesterol biosynthetic pathway (Figure 1.3 – 2). They are 27-carbon molecules generated either by enzyme-catalyzed or non-enzymatic mechanisms. The most important oxysterols are generated by cholesterol hydroxylases, located in mitochondria or endoplasmic reticulum and belonging to the cytochrome P450 family (Luoma, 2007; Russell, 2000). Oxysterols fall into two main categories:

- 1) Species oxygenated on the sterol ring, mainly at the C7-position (e.g., 7 $\alpha$ / $\beta$ -hydroperoxycholesterol (7OOHC), 7-ketocholesterol (7-KC) and 7 $\alpha$ / $\beta$ -hydroxycholesterol (7OHC)). These compounds form the focus of the work described in this thesis
- 2) Species oxygenated on the side-chain (e.g., 24S-hydroxycholesterol (24S-OHC), 25-hydroxycholesterol (25-OHC) and 27-OHC).

Generally, ring-oxygenated sterols tend to be formed non-enzymatically, whereas side-chain oxygenated sterols tend to be of an enzymatic origin. However, some ring oxysterols, previously defined as auto-oxidation products, are also formed via enzymatic means, as recently reported (Gill *et al.*, 2008; Hult *et al.*, 2004). Although oxysterols are less than 0.1 % of the total cholesterol in the body, they are major components of oxLDL (~60 %) and are, hence, highly abundant in the plasma and vessel wall (Brown and Jessup, 1999; Dzeletovic *et al.*, 1995a).

7-Oxygenated sterols, namely 7-keto- (7-KC), 7 $\alpha$ -hydroxy- (7 $\alpha$ OH) and 7 $\beta$ -hydroxy- (7 $\beta$ OH) cholesterol can also be formed non-enzymatically by direct radical attack on the cholesterol C7 position by reactive oxygen species (ROS) such as the hydroxyl radical. Formation of a carbon-centred radical at the C7 position produces a long-lived and reactive form, which can further lead to generation of the cholesterol peroxy radical (COO $\cdot$ , Figure 1.3 – 3). Further abstraction of hydrogen from a second lipid molecule results in relatively stable hydroperoxy cholesterol (7 $\alpha$ -/7 $\beta$ -OOHC).

Biologically active oxysterols may also be generated by enzymatic routes, most pertinently for this thesis (see section 1.4) through metabolism by 11 $\beta$ HSD1 (see Figure 1.3 – 3).



**Figure 1.3-3 The reaction mechanism leading to the production of 7-oxygenated cholesterol derivatives.**

Cholesterol oxidation on position C7 is initiated by a radical attack forming a delocalized three-carbon allylic radical, which reacts with oxygen ( $O_2$ ) to produce the epimeric products 7 $\alpha$ - and 7 $\beta$ -hydroperoxy-cholesterol. This species can decompose to hydroxy and keto analogues, 7 $\alpha$ -hydroxycholesterol (7 $\alpha$ OHC), 7 $\beta$ -hydroxycholesterol (7 $\beta$ OHC) and 7-ketocholesterol (7-KC). Human and rodent 11 $\beta$ -hydroxysteroid dehydrogenase type 1 (11 $\beta$ HSD1) interconvert 7 $\beta$ OHC and 7-KC, whereas hamster 11 $\beta$ HSD1 reduces 7-KC to 7 $\alpha$ OHC and/or 7 $\beta$ OHC.

### 1.3.2. Targeted Actions Of 7-Oxysterols

Oxysterols have multiple, often poorly-understood biological effects (summarized in Table 1 - 1; (Bjorkhem and Diczfalusy, 2002; Schroepfer, 2000)). Some key biological actions of 7-oxysterols are described further:

#### 1.3.2.1. *Apoptosis And Necrosis*

Apoptosis (programmed cell death) is a fundamental biological process that is highly regulated and controlled by the cell. It was first described by Kerr and colleagues (Kerr *et al.*, 1972) and involves two pathways: extrinsic (involving death receptors (Fas)) and intrinsic (involving mitochondria). Apoptotic cells are recognized for non-inflammatory clearance by phagocytes (e.g. macrophages) and this process has implications for all stages of atherogenesis and plaque rupture (Kavurma *et al.*, 2005). 7-Oxysterols may induce death in various cell types by these mechanisms (including those found in the vascular wall). Indeed, both apoptosis and necrosis have been attributed to exposure to either, or a combination of, 7-KC or 7 $\beta$ OHC (Lizard *et al.*, 1999). The proposed mechanism(s) through which oxLDL and the oxysterol component of oxLDL cause cell death was described recently (Panini and Sinensky, 2001). An elevation in intracellular calcium levels is an early signal transduction event that leads to apoptosis via extrinsic and intrinsic pathways. In particular, 7-KC-induced apoptosis involves calcium-dependant activation of pro-apoptotic and survival pathways (Berthier *et al.*, 2005) by increasing the influx of calcium into, for example, endothelial cells (Zhou *et al.*, 2000). 7-KC-induced apoptosis of vascular smooth muscle cells is not caspase-dependent but occurs as a result of a mitochondrial conformational change rather than swelling and rupture of the outer membrane (Seye *et al.*, 2004). In contrast, 7 $\beta$ OHC-induced apoptosis is thought to occur via activating Fas ligand (FasL) leading to stimulation of the Fas receptor (FasR) (Lordan *et al.*, 2008). The process of apoptosis induced by 7-KC or 7 $\beta$ OHC in the vascular environment can be reversed by the addition of an antioxidant such as lycopene (Palozza *et al.*, 2009), which also prevents 7-KC-induced oxidative stress.

**Table 1-1 Major biological functions of oxysterols.**

<b>BIOLOGICAL FUNCTION OF OXYSTEROLS</b>	<b>References:</b>
Bile acid and steroid hormone synthesis	(Bjorkhem, 2002; Chiang, 2002; Kalaany and Mangelsdorf, 2006)
Cellular sterol efflux	(Gelissen et al., 1996; Huang et al., 2001; Jessup and Kritharides, 2008)
Cytotoxic and pro-apoptotic effects (e.g. 7-ketocholesterol induces cell apoptosis)	(Asano et al., 2007; Vejux et al., 2007)
Pro-inflammatory signals (e.g. 7 $\beta$ -hydroxycholesterol can induce secretion of interleukin IL-1 $\beta$ in vascular endothelial cells)	(Lemaire-Ewing et al., 2005; Rosklint et al., 2002)
Regulation of lipid metabolism via liver-X-receptor (LXR) and Insig (a sterol response element binding protein) – (e.g. some oxysterols bind to LXRs; LXR $\beta$ can regulate actions of glucocorticoids and is a potential target for drug treatment of diabetes)	(Brown et al., 2002; Fu et al., 2001; Kalaany and Mangelsdorf, 2006)
Modulation of estrogen receptor (ER) function – (e.g. 27-hydroxycholesterol binds to and activates ER $\beta$ , instead of estrogen)	(Umetani et al., 2007) (DuSell et al., 2008)
Regulation of lipid metabolism, vesicle transport and signalling via OSBP (oxysterol-binding protein) and ORPs (OSBP-related proteins)	(Suchanek et al., 2007) (Yan et al., 2008)
Activators of Hedgehog signalling, cell differentiation (e.g. oxysterols stimulate hedgehog pathway activity via indirect activation of Smoothened, thus regulating developmental processes; and can increase formation of medulloblastoma).	(Corcoran and Scott, 2006; Dwyer et al., 2007)

Oxysterols are synthesized from cholesterol, as their major precursor, by the actions of Cytochrome P450s and exert numerous biological functions (Olkonen and Hynynen, 2009).

### **1.3.2.2. Actions via Nuclear Receptors And Regulation Of Cholesterol Efflux And Fat Metabolism**

Nuclear receptors (NRs) integrate metabolic pathways, including those involved with cholesterol homeostasis ([www.nursa.org](http://www.nursa.org)), and are transcription factors (TF). They bind to a target gene region and the transcriptional outcome depends on their assembly with associated co-activators and co-repressors. NRs target a network of genes in response to extracellular stimuli, and often respond to small hydrophobic ligands such as oxysterols (Hult *et al.*, 2004). Nuclear hormone receptors are thought to aid maintenance of allostasis (i.e. energy supply and lipid composition), which was defined by Sterling and Eyer (2002) as an adaptive process for effectively maintaining cell stability and balance. NRs have a conserved structure comprising a DNA-binding domain (DBD) and a ligand-binding domain (LBD). The ligands for nuclear hormone receptors are often lipophilic hormones.

Some nuclear hormone receptor agonists are promising drugs as they: can promote cholesterol efflux; can increase HDL levels; have anti-inflammatory activity and can reduce progression of atherosclerosis in mice (Joseph and Tontonoz, 2003; Kalaany and Mangelsdorf, 2006). Many oxysterols have been shown to activate various nuclear receptors. Most recently 27-OHC has been named an endogenous selective modulator of estrogen receptor  $\beta$  (SERM) via alteration of ER structure and cofactor recruitment (DuSell *et al.*, 2008).

The liver X receptor (LXR)-pathway is recognized as a potential target for therapeutic intervention in human cardiovascular disease, since synthetic LXR agonists inhibit the development of atherosclerosis in murine models (Tontonoz and Mangelsdorf, 2003). LXRs are small orphan receptors, critical for the control of lipid homeostasis in vertebrates, expression of various genes involved in the efflux, transport and excretion of cholesterol. LXR $\alpha$  (NR1H3, From the Nuclear Receptors Nomenclature Committee, (1999)), but not LXR $\beta$  (NR1H2), is mainly important in cholesterol homeostasis in the liver. LXR $\beta$ , similar to LXR $\alpha$ , is involved in the regulation of cholesterol metabolism in peripheral tissues (Alberti *et al.*, 2001).

Recent studies have identified several oxysterols, in particular 24(S)-, 25-epoxycholesterol, and 24(S)-hydroxycholesterol (Lehmann *et al.*, 1997), as natural ligands for LXR $\alpha$  receptor. 7 $\alpha$ OHC can stimulate LXR $\alpha$ -dependent transcription, although to a lesser extent than 22(R)-hydroxycholesterol (Janowski *et al.*, 1999). 7-KC behaves as a very weak agonist for the LXR family, and upon activation of LXR-mediated transcription by the synthetic non-steroidal ligand T0901317 7-KC acted also as a partial agonist (Crestani *et al.*, 2004). More recently, 7-KC and 7 $\beta$ OHC have been ruled out as agonists for this receptor since they failed to activate LXR in mature adipocytes, or to display intrinsic activation of the glucocorticoid receptor (Wamil *et al.*, 2008).

7-KC, a physiological stimulus, has (with a number of xenobiotics) been proposed to modulate activation of the aryl hydrocarbon receptor (AhR) (Savouret *et al.*, 2001). The ligands for this receptor have been implicated in the development of cardiovascular diseases due to their oxidative and atherogenic properties to activate or mediate activity of AhR in the vascular endothelium (Crawford *et al.*, 1997; Dabir *et al.*, 2008). Although it is not an agonist for this receptor, 7-KC could competitively bind to the AhR and function as an AhR antagonist, but other 7-oxysterols do not exert this characteristic action (Crawford *et al.*, 1997).

### 1.3.3. The Pathophysiological Role Of 7-Oxysterols

Plasma levels of both 7 $\beta$ OHC and 7-KC have been proposed as biomarkers for a number of conditions, including: cancer, oxidative stress and inflammation in diabetes (Linseisen *et al.*, 2002). Jessup and Brown reported increased circulating concentrations of 7-oxysterols in patients with atherosclerosis (Brown and Jessup, 1999, 2009; Jessup and Brown, 2005) and these compounds possess a number of functions that have linked them with the pathogenesis of cardiovascular disease. These include their: pro-oxidative, pro-inflammatory (Rosklint *et al.*, 2002), pro-coagulant (Aupeix *et al.*, 1996) and pro-apoptotic properties (Berthier *et al.*, 2005; Prunet *et al.*, 2006) allied with their ability to induce both oxidative stress in the cells of vascular wall and (Olkkonen and Lehto, 2004) cell death in atherosclerosis



(Akishima *et al.*, 2005). The 7-hydroxyperoxides and 7-oxysterols are major components (up to 40%) of LDL particles. Furthermore, high concentrations of 7-oxysterols ( $\geq 100$ -fold higher than in plasma) are found in human atherosclerotic plaques (Bjorkhem *et al.*, 1994; Zieden *et al.*, 1999). In particular, 7-KC is a major 7-oxysterol found in atherosclerotic plaques (Brown *et al.*, 1997; Suarna *et al.*, 1995), and many reviews (Berliner and Heinecke, 1996; Bjorkhem and Diczfalussy, 2002; Brown and Jessup, 2009; Lukyanenko and Lukyanenko, 2009; Olkkonen and Lehto, 2005) have discussed the role of 7-KC and 7 $\beta$ OHC in atherosclerosis due to both their high abundance in atherosclerotic plaques and their ability to trigger pro-apoptotic events.

More recently oxysterols have been identified as major regulators of cholesterol metabolism (Wang *et al.*, 2008). They can also modulate activity of the estrogen receptor and recruitment of its cofactor (DuSell *et al.*, 2008). Oxysterols are potent membrane destabilizing agents, as they are able to displace cholesterol from its major sites (within cell membranes, next to phospholipids; (Siggins *et al.*, 2006)). Cholesterol is (chemically) less susceptible to oxidative attack than the polyunsaturated fatty acyl moieties present in phospholipids. This is illustrated by the more extensive oxidation of fatty acyl groups compared with cholesterol in isolated LDL (Noguchi *et al.*, 1998). Oxidized lipids can, however, cross the cell membrane easily and equilibrate with lipoproteins at a much faster rate than cholesterol (Massey, 2006). Along with the biological effects described (above), oxysterols can inhibit or stimulate important enzymes in cholesterol homeostasis and exert mutagenic effects. For example, oxidized cholesterol can prevent synthesis of enzymes linked to sterol metabolism, at an mRNA level. One such example is the inhibition of HMG-CoA reductase (the rate-limiting enzyme in cholesterol synthesis), thereby leading to reduced endogenous cholesterol synthesis (Schmidt *et al.*, 2006). Oxysterols also have cytotoxic and apoptotic effects on the (endothelial and smooth muscle) cells of the vascular wall (Lizard *et al.*, 1999), and (along with oxLDL) are susceptible to incorporation by macrophages, leading to foam cell formation (Hayden *et al.*, 2002).

In terms of atheroma, formation of oxysterols is considered a way of eliminating excess cholesterol via inhibition of its synthesis (by HMG-CoA). As the work by Terasaka *et al.*, (2007), elegantly demonstrated, ATB-binding cassette transporters may aid export of 7-oxysterols from macrophages. In particular, over-expression of the ABCG1 transporter promotes cellular efflux of 7-KC and 7 $\beta$ OHC to HDL acceptor particles. This could favour prevention of atherosclerotic plaque formation and suggests a metabolic benefit of increased local 7-oxysterols as opposed to cholesterol.

#### **1.3.3.1. The Endothelium Is A Target For 7-Oxysterols**

Although the formation of oxysterols aids clearance of excess cholesterol via formation of bile acids, there is increasing evidence that 7-oxysterols impair the function of vascular endothelial and smooth muscle cells. The precise mechanism(s) involved, however, are not completely understood. A better understanding of these actions should clarify the processes underlying 7-oxysterol-mediated impairment of endothelium-dependent vasorelaxation (Deckert *et al.*, 1997). One potential mechanism may involve 7-oxysterol-mediated cell death as both 7 $\beta$ -hydroxycholesterol and 7-ketocholesterol have been shown to reduce cell adhesion and increase permeability to propidium iodide in endothelial cells, smooth muscle cells and fibroblasts (Lizard *et al.*, 1999). Furthermore, 7-oxysterols may also induce apoptosis via a nitric oxide (NO)-dependent pathway (Panini and Sinensky, 2001). This is consistent with the demonstration that increased dietary oxysterols are toxic to the endothelium and smooth muscle cells *in vivo*, through the release of cytochrome C (although this toxicity was counterbalanced by increased endothelium-dependent dilatation (Meynier *et al.*, 2005)). These results suggest that detrimental effects of 7-oxysterols on endothelial cell function may contribute to the development of cardiovascular conditions such as atherosclerosis. Consequently, regulation of 7-oxysterol concentrations in the vascular wall may have a considerable influence on pathogenesis of these conditions.

## 1.4. Novel Function For 11 $\beta$ HSD1 In 7-Oxysterol Metabolism

In addition to its metabolism of glucocorticoids, recent reports (using different animal models) have suggested a novel role for 11 $\beta$ HSD1 in the inter-conversion of 7-oxysterols (as mentioned in see sections 1.2.1 and 1.3.2). Several research groups have shown that 11 $\beta$ HSD1 plays a role in formation of 7-KC from 7 $\beta$ -hydroxycholesterol (Hult *et al.*, 2004) and that 11 $\beta$ HSD1-mediated reduction of 7-KC occurs in the (rat) liver (Schweizer *et al.*, 2004). These studies on metabolism of 7-oxysterols by 11 $\beta$ HSD1 have been performed using enzyme from a variety of species (rat, rabbit, guinea pig, chicken, dog and human). In all species 11 $\beta$ HSD1 converted 7-KC to 7 $\beta$ OHC but in some species 7 $\alpha$ - and/or 7 $\beta$ OHC were also converted into 7-KC. The dehydrogenation of 7 $\beta$ OHC was only specific to hepatic 11 $\beta$ HSD1 in the hamster (Arampatzis *et al.*, 2005). Still, the preferential direction and the specificity of 11 $\beta$ HSD1 for 7-oxysterols remain unclear and it has been proposed that the specificity of 11 $\beta$ HSD1 for 7-oxysterols should be tested in an animal lacking the enzyme (Jessup and Brown, 2005). The contribution of 11 $\beta$ HSD1 to the formation of 7-oxysterols may also be considered a mechanism for removal of excess 7-KC. The contribution of 11 $\beta$ HSD1 to regulation of 7-oxysterols in atherosclerotic plaque has not been addressed.

### 1.4.1. Research That Identified A Novel Function For 11 $\beta$ HSD1

The existence of an additional unidentified pathway for metabolism of 7-KC in mice initially became evident from the work by Erikson *et al.*, (1977). This identified 7 $\beta$ -hydroxycholesterol as a major metabolite upon hepatic perfusion with [ $^3$ H]-7-KC in rats. More recently Lyons *et al.*, (2002) proposed that metabolism of 7-KC in the liver would solely be catalysed by 27-hydroxylase. However, by performing work in mice carrying a homozygous null mutation in CYP27 (Cyp27 $^{-/-}$ ) the same group detected an *increased* rate of metabolism of 7-KC (compared with wild type controls) to an unknown product, as opposed to the *reduced* rate that they anticipated. This was evidence that mice do not have an absolute requirement for

27-hydroxylase in order to metabolise 7-KC, but that an alternative route exists. Earlier work by Song *et al.*, (1998) is consistent with this view, with the demonstration that NADP<sup>+</sup>-dependent metabolism of 7 $\beta$ OHC in hamster microsomes generated 7-KC. They also showed that the enzyme involved was a type of HSD (“7 $\alpha$ HSD”) and reported its ability to metabolise oxysterols and glucocorticoids (e.g. hamster, *K<sub>m</sub>* for corticosterone, 1.2  $\mu$ M and for 7 $\beta$ OHC, 1.9  $\mu$ M, with identical *V<sub>max</sub>*, ~170 nmol/min/mg protein). Further cloning and expression in a cell system identified this enzyme as 11 $\beta$ HSD1; probably representing the major hepatic route of 7-KC metabolism in the body (Hult *et al.*, 2004). Subsequent work (Schweizer *et al.*, 2004) confirmed that inhibition of 11 $\beta$ HSD1 by carbenoxolone abolished the formation of this 7-oxysterol metabolite by rat hepatic microsomes. This was the first report of the metabolic specificity of 11 $\beta$ HSD1 for the 7-oxysterol pathway but the preferred substrate and the reaction equilibrium for this pathway were not determined. Further work has demonstrated clear inter-species differences in 11 $\beta$ HSD1-mediated metabolism of 7-KC. The hamster enzyme differs fundamentally from 11 $\beta$ HSD1 in rat, human or guinea pig in that it accepts both 7-epimers (7 $\beta$ OHC and 7 $\alpha$ OHC) as substrates for the dehydrogenase reaction. Equally, reduction of 7-KC produces 7 $\beta$ OHC and/or 7 $\alpha$ OHC in the hamster. In rat and guinea pig, however, only 7 $\beta$ OHC is accepted as a substrate or formed following reduction of 7-KC (Hult *et al.*, 2004). Inter-conversion of 7 $\beta$ OHC and 7-KC has also been demonstrated recently in humans *in vivo*. Administration of deuterium-labelled isomers of 7 $\beta$ OHC and 7-KC (d7-7 $\beta$ OHC, d7-7-KC) to healthy volunteers, resulted in their direct inter-conversion (Larsson *et al.*, 2007), which the authors ascribed to the action of 11 $\beta$ HSD1. Although not tested directly, this is the first report on the role of human 11 $\beta$ HSD1 in determining circulating levels of 7-oxysterols.

#### 1.4.2. Different Substrates Exist For 11 $\beta$ HSD Type1

The main function attributed to 11 $\beta$ HSD1 *in vivo* is to activate inert 11-keto glucocorticosteroids (e.g. cortisone, corticosterone) and form active hormones (e.g. cortisol, 11-dehydrocorticosterone). However, from the evidence published by 2005, it became clear that 11 $\beta$ HSD1 might be involved both in oxysterol and in glucocorticoid metabolism, concomitantly. This dual metabolism of 11 $\beta$ HSD1

suggests that there are factors common to both classes of substrates which regulate these two pathways in the body. Indeed, conditions of glucocorticoid excess are commonly associated with dyslipidaemia (Morton *et al.*, 2001). However, the specificity of 11 $\beta$ HSD1 for, and the physiological consequences of, the interaction between these two pathways has not been determined or compared in different species. Indeed, enzymatic formation of 7-oxysterols (7-KC and 7 $\beta$ OHC) by 11 $\beta$ HSD1 only recently attracted more attention following the suggestion that 11 $\beta$ HSD1 inhibition had the potential to prevent atherosclerosis and hyperlipidaemia (Hermanowski-Vosatka *et al.*, 2005). Whilst the role for 11 $\beta$ HSD1 in metabolizing glucocorticoids is well established, the physiological significance of the novel pathway of 7-oxysterol metabolism is not yet clear, as the functions of the substrate and product of 11 $\beta$ HSD1-mediated 7-oxysterol metabolism are less well delineated. Generation of glucocorticoids by 11 $\beta$ HSD1 is a potential pharmacological target for several metabolic diseases. Whether the same, or additional, benefits can be obtained by inhibiting 11 $\beta$ HSD1-mediated metabolism of 7-oxysterols is not yet known.

It is clarification of the interaction between glucocorticoids, 7-oxysterols and 11 $\beta$ HSD1 that forms the basis of the work presented in this thesis. Confirmation of the physiological and pathophysiological roles of glucocorticoid metabolism by 11 $\beta$ HSD1 has been greatly aided by the generation (in Edinburgh; Kotelevstev *et al.*, 1997) of mice with selective transgenic deletion of this enzyme. Determining whether 7-oxysterols also act as substrates for murine 11 $\beta$ HSD1, would make it possible to use these knockout mice to clarify the importance of 11 $\beta$ HSD1-mediated inter-conversion of 7-oxysterols in health and disease.

## 1.5. Hypothesis And Aims

The experimental work performed and presented in this thesis was based on the finding that 7-oxysterols are metabolised by the enzyme 11 $\beta$ HSD1 in rodents. However, there was little evidence to suggest this function was conserved in murine 11 $\beta$ HSD1: this was a significant omission because much mechanistic work has been performed in mice with transgenic deletion of this enzyme. In addition it was not clear whether oxysterols compete with glucocorticoids for the active site of 11 $\beta$ HSD1 (with consequent implications for metabolism of both classes of substrate. Consequently, the work described in this thesis addresses the hypothesis that murine 11 $\beta$ HSD1 enzyme metabolises 7-oxysterols; which may have direct physiological consequences but also may modulate the activity of glucocorticoids.

In order to investigate this hypothesis the following aims were set:

1. To investigate the kinetics of 11 $\beta$ HSD1-mediated metabolism of 7-oxysterols using mouse recombinant protein and mouse tissue homogenates.
2. To determine whether 7-oxysterols and glucocorticoids compete for metabolism by 11 $\beta$ HSD1.
3. To determine whether cellular cholesterol levels *in vitro* influence 7-oxysterol levels and the kinetics of glucocorticoid metabolism by 11 $\beta$ HSD1.
4. To assess the impact of genetic deletion (or inhibition) of 11 $\beta$ HSD1 in mice, on the plasma levels of 7-oxysterols.
5. To determine whether exposure to 7-oxysterols alters the function of isolated mouse aorta.

## **Chapter 2. Materials and Methods**

All solvents and water were glass-distilled HPLC grade purchased from Fisher (Loughborough, UK) or from Rathburn (Walkerburn, UK). All radioactively-labelled species were purchased from GE Healthcare (Bucks, UK) and scintillation fluid GoldFlow, was from Meridian (Surrey, UK). All chemicals were from Sigma-Aldrich (Gillingham, UK) or Fluka (Buchs, Switzerland) unless otherwise stated. Tissue culture reagents were from Lonza (Vervier, Belgium) and plastics from Costar Ltd (Corning®, Sigma) or from BD Falcon (BD Biosciences, Bedford, USA).

## 2.1. Chemicals

5-Cholesten-3 $\beta$ -ol-7-one (7-Ketocholesterol), 5-cholesten-3 $\beta$ ,7 $\beta$ -diol (7 $\beta$ -hydroxycholesterol), 5-cholesten-3 $\beta$ ,7 $\alpha$ -diol (7 $\alpha$ -hydroxycholesterol); 4-cholesten-7 $\alpha$ -ol-3-one (C4), 5-cholesten-3 $\beta$ ,19-diol (19-hydroxycholesterol), 11 $\alpha$ -hydrocortisone (epi-F), 11-dehydrocorticosterone (A) and corticosterone (B) were purchased from Steraloids (Newport, Rhode Island, USA). 5-Cholesten-3 $\beta$ ,27-diol (27-hydroxycholesterol) was purchased from Avantis Polar Lipids (Alabama, USA). Hepta-deuterated isotopomers, d7-7 $\beta$ OHC (7 $\beta$ -hydroxycholesterol – 25, 26, 26, 26, 27, 27, 27-d<sub>7</sub>), d7-7-KC (7-Ketocholesterol – 25, 26, 26, 26, 27, 27, 27-d<sub>7</sub>) and d7-cholesterol (cholesterol – 25, 26, 26, 26, 27, 27, 27-d<sub>7</sub>) used as internal standards, were purchased from CDN Isotopes (Quebec, Canada).

## 2.2. Buffers

All buffers were prepared to give final concentrations as stipulated in brackets and pH was adjusted as appropriate.

**‘C’ buffer for preparation of [<sup>3</sup>H]<sub>4</sub>-A:** NaCl (150 mM), EDTA (1 mM), Tris (50 mM) were dissolved in water and the pH adjusted to 7.7 with concentrated NaOH, before glycerol was added (8%, v/v).



**High Potassium-physiological saline solution (KPSS):** KCl (125 mM), MgSO<sub>4</sub> (1.17 mM), NaHCO<sub>3</sub> (25 mM), KH<sub>2</sub>PO<sub>4</sub> (1.18 mM), K<sub>2</sub>EDTA (0.03 mM), CaCl<sub>2</sub> (2.5 mM), D-glucose (5.5 mM) were dissolved in water and the pH adjusted to 7.4 with concentrated NaOH.

**Homogenising buffer for placental tissue:** 'C' buffer as above was used, to which dithiothreitol (DTT, 7.7 mg/50 ml) was added.

**Krebs–Ringer bicarbonate buffer (Krebs):** NaCl (118 mM), KCl (3.8 mM), KH<sub>2</sub>PO<sub>4</sub> (1.19 mM), CaCl<sub>2</sub> (2.54 mM), MgSO<sub>4</sub> (1.19 mM), NaHCO<sub>3</sub> (25 mM) were dissolved in water and the pH was adjusted to 7.4 with concentrated NaOH.

**Potassium Phosphate Buffer (Phosphate Buffer):** Final solution (0.1M, 1 L) was prepared from two stock solutions of KH<sub>2</sub>PO<sub>4</sub> (1 M, 19.8 ml) and K<sub>2</sub>HPO<sub>4</sub> (1 M, 80.2 ml) in water, to which EDTA (0.1 M) was added and the pH adjusted to 7.5 using KOH pellets.

**Phosphate Buffered Saline (PBS)** was prepared using Potassium Phosphate Buffer as above, with NaCl (137 mM) and KCl (2.7 mM). The pH was adjusted to 7.4 with concentrated NaOH and the solution was sterilized in an autoclave before use.

**Physiological saline solution (PSS)** was prepared using NaCl (119 mM), KCl (4.7 mM), CaCl<sub>2</sub> (2.5 mM), MgSO<sub>4</sub> (1.17 mM), KH<sub>2</sub>PO<sub>4</sub> (1.18 mM), K<sub>2</sub>EDTA (0.026 mM) and D-glucose (5.5 mM). The pH was adjusted to 7.4 with concentrated NaOH.

**Recombinant protein (Tris) extraction buffer:** NaCl (300 mM), Tris (50 mM), Triton-X100 (0.05%, v/v) were dissolved in water and the pH adjusted to 8.0 with concentrated NaOH.

**Sodium Phosphate Buffer:** Final solution (40 mM, 1L) was prepared from two stock solutions of NaH<sub>2</sub>PO<sub>4</sub> (0.2M, 46 ml) and Na<sub>2</sub>HPO<sub>4</sub> (0.2 M, 154 ml) in water to which glycerol (8 %) was added and the pH adjusted to 7.4 using KOH pellets.

**Sucrose buffer for microsome preparation:** sucrose (0.25M) was dissolved in water and the pH adjusted to 7.5 with concentrated NaOH.

**Tris NaCl assay buffer:** Tris-HCl (50 mM) and NaCl (50 mM) were dissolved in water and the pH adjusted to 7.0 with concentrated NaOH.

**TG1 lysis buffer:** NaCl (100 mM), EGTA (1 mM), EDTA (1 mM), MgCl<sub>2</sub> (1 mM), glycerol (20%, v/v), Tris-HCl (20 mM) were dissolved in water and the pH was adjusted to 7.4 with concentrated NaOH.

### 2.2.1. Drugs And Buffers Used In Myography Studies

**Acetylcholine (ACh, chloride salt):** ACh (54.51 mg) was dissolved in water (30 ml,  $10^{-2}$  M) and stored in aliquots (500  $\mu$ l).

**5-Hydroxytryptamine (5HT):** 5HT (11.62 mg) was dissolved in water (30 ml,  $10^{-2}$  M) and stored in aliquots (1 ml).

**Noradrenaline (NA, bitartrate salt):** NA (31.93 mg) was dissolved in water (10 ml,  $10^{-2}$  M) and stored in aliquots (500  $\mu$ l).

**Potassium Chloride (KCl):** KCl (14.91g) was dissolved in water (100 ml, 2M) and stored at 4°C.

**Sodium Nitroprusside (SNP):** SNP (50 mg, pharmaceutical grade, David Bull Laboratories, Faulding, UK) was dissolved in water (500 ml), NaCl (0.9% v/v) was added and stock ( $10^{-2}$  M) stored in aliquots (500  $\mu$ l).

**DMEM-L-Arginine:** Medium, DMEM without L-arginine was custom made by Lonza (Vervier, Belgium).

**KPSS – DMEM-L-Arginine:** KCl (123.7 mM) was freshly dissolved in the DMEM without phenol red or L-arginine, mixed and oxygenated (95% O<sub>2</sub> and 5% CO<sub>2</sub>, 37°C).

### 2.2.2. Storage And Preparation Of Reagents And Buffers

- All buffers were stored at 4°C.
- All extraction solvents contained an antioxidant, butylated hydroxytoluene (BHT), at a final concentration of 50  $\mu$ g/ml. The stock solution of BHT was prepared in ethanol (10 mg/ml).
- HPLC grade solvents were all stored at room temperature and mixed in appropriate ratios or individually filtered (0.45 $\mu$ m HV membrane filters, Millipore Ltd, Watford, UK) before use in HPLC analysis.
- Reacti vials were silanised in trichloromethylsilazine (TMCS) in toluene (5%) by sonication (10 min), then rinsed first with pure toluene followed by methanol, before they were air-dried (at RT).

- Cofactors, chemical powders, stock solutions and dilutions of unlabelled and tritiated oxysterols and steroids were stored at -20°C, except for unlabelled 'A' which was kept away from light (wrapped in tin foil) and stored at -80°C. Subsequent dilutions of 'A' were prepared on the day. Cofactors were always dissolved in the relevant assay buffer for specific reactions.
- Sterol, mixture of sterol and d<sup>7</sup>-sterol solutions (4 mg/ml, 10 mM) were prepared in ethanol containing BHT (50 µg/ml) as an antioxidant (Schroepfer, 2000). The vials were sealed under argon, sonicated (on ice, 10 min), then vortexed (400 rpm, RT, 10 min) until fully dissolved and stored in aliquots (500 µl) at -20°C. Dilutions were prepared in ethanol, under argon gas: vials were sealed and stock solutions stored at -20°C. If vials were opened more than three times, stocks were discarded. During the assay preparation all dilutions of sterols were kept on dry ice to minimize exposure to the air.
- Reagents for GC/MS derivatisation were kept away from light and moisture, and stored in amber glass containers under argon and in a dessicator.
- In all experiments room temperature (RT) was 18 – 22°C.

## **2.3. General Lab Equipment**

- A Beckman Optima TLX Ultracentrifuge (GMI Inc, Ramsey, USA) was used for preparation of microsomes.
- Separation of recombinant protein pellets was carried out in a Beckman J2-MC high-speed centrifuge (GMI Inc, Ramsey, USA).
- Centrifugal steps requiring <3,000 g were carried out in a Heraeus labofuge-400 (Thermo Fisher Scientific Inc, Waltham, USA).
- Homogenization of tissues was carried out using a Pro200 homogenizer (ProScientific, Oxford, USA).
- Samples for some of the GC/MS procedures were derivatized in a Memmert D88 oven (Memmert, Nürnberg, Germany).
- Aliquots of radiolabelled samples were analysed using Tri-carb Liquid Scintillation Counter (Packard, Global Medical Instrumentation, Ramsey, USA).

- Cell monitoring for tissue culture work was observed under light microscope (x40, Zeiss, Hertfordshire, UK).
- Danish Myo-Technology 4-channel myographs (700MO) attached to Powerlab v4.0 data acquisition software were used for functional studies with mouse aortae.

## 2.4. Animal Maintenance And Tissue Harvesting

The following control and transgenic animals were used in the experiments described in this thesis. All transgenic animals were generated on, or back-crossed (twelfth-generation) onto, a C57Bl/6 background:

- C57Black/6 mice (in-house stock, or from Harlan Ltd, Oxon, UK);
- 11 $\beta$ HSD1 deficient mice (11 $\beta$ HSD1<sup>-/-</sup> (Kotelevtsev *et al.*, 1997; Morton *et al.*, 2004) in-house colony);
- 11 $\beta$ HSD2 deficient mice (11 $\beta$ HSD2<sup>-/-</sup> (Kotelevtsev *et al.*, 1999) in-house colony);
- ApoE knockout mice (ApoE<sup>-/-</sup> (Plump *et al.*, 1992; Zhang *et al.*, 1992) in-house colony);
- 5 alpha reductase-1 (SRD5A1) heterozygous mice (5 $\alpha$ SRD1<sup>+/-</sup>, Mahendroo *et al.*, (1997) , Mahendroo *et al.*, (2001), in-house colony);
- Wistar rats (Harlan Ltd, Oxon, UK).

All animals were bred at the Biomedical Research Facility (BRR, Little France, Edinburgh and at the BRR Western General Hospital, Edinburgh). All animals were maintained on standard chow diet and tap water ad libitum, under the 16/8 (light/dark) cycle; room temperature was 19.5 - 23.5°C.

All experimental procedures were performed under a UK Home Office Project Licence (Seckl, 60/3293) and personal licence (Mitić, 60/10525).

### 2.4.1. Tissue Collection

All animals were sacrificed by decapitation or asphyxiation as required by experimental conditions. The standard procedure for tissue collection involved removing the liver, kidney and aorta. Liver and kidneys were dissected from the

animals, wrapped in aluminium foil, placed on dry ice then stored at  $-80^{\circ}\text{C}$ . Small amounts of liver ( $\sim 0.1$  g) were stored and kidney samples were cut in half. Aortic tissue was processed as described in section 2.4.5.

### **2.4.2. Hepatic Subcellular Fractionation**

Livers were stored at  $-80^{\circ}\text{C}$  until use when they were slowly defrosted on wet ice. Each sample was cut into small pieces (0.1 g) and disrupted repeatedly (15 sec) in glass tubes (10x1cm) containing sucrose buffer (1 ml). Tubes were stored on wet ice. Homogenates were transferred into further glass tubes (13x1cm) and sucrose buffer was added to 90% tube height. Samples were subjected to centrifugation (1000 g,  $4^{\circ}\text{C}$ , 15 min) for separation of the nuclear fraction (pellet) and the supernatant was stored on wet ice and then transferred into Beckman ultracentrifuge tubes (5x1cm). A further centrifugal separation (13,000 g,  $4^{\circ}\text{C}$ , 30 min) was performed. The subsequent supernatant was collected in new Beckman tubes and another centrifugal separation was performed (23913 g,  $4^{\circ}\text{C}$ , 1h) to obtain light microsomes (pellet) and cytosolic fractions (supernatant). The supernatant was stored at  $-80^{\circ}\text{C}$ . The remaining pellet of microsomes was re-constituted in sucrose buffer (500  $\mu\text{l}$ ), aliquots (40  $\mu\text{l}$ ) removed for immediate quantitation of protein (as in section 2.4.7) and remaining samples were stored at  $-80^{\circ}\text{C}$ .

### **2.4.3. Renal Homogenates**

Kidneys ( $\sim 0.5$  g) were kept on ice at all times and homogenized in Krebs or Sucrose buffer (1ml, on ice), then further diluted in Phosphate Buffer. Tubes containing homogenates were subject to centrifugation (500 g,  $4^{\circ}\text{C}$ , 2 min) and the supernatant harvested. Aliquots (10  $\mu\text{l}$ ) were removed immediately for determination of protein concentration (section 2.4.7) and the remaining samples frozen at  $-80^{\circ}\text{C}$ , and only defrosted once.

#### **2.4.4. Placentae**

Placentae (a kind gift from Ms Lynne Ramage, Endocrinology, University of Edinburgh) were collected from female Wistar rats (Harlan Ltd, Oxon, UK) after cull, rinsed in PSS buffer, frozen immediately on dry-ice and stored at  $-80^{\circ}\text{C}$ . Two whole placentae were homogenized in the cold buffer (1 ml) and kept on wet ice at all times. Homogenates were stored in aliquots (500  $\mu\text{l}$ ) at  $-80^{\circ}\text{C}$  and only defrosted once.

#### **2.4.5. Intact Aortic Preparations**

The thoraco-abdominal aorta was removed quickly post-mortem into cold ( $4^{\circ}\text{C}$ ) PSS, then transferred to cold ( $4^{\circ}\text{C}$ ) oxygenated (95%  $\text{O}_2$ /5%  $\text{CO}_2$ ) PSS solution and dissected. Fresh aortae were gently handled using fine dissecting tools (FST, Inter focus Ltd, Cambridge, UK) under a dissection microscope (x20, Zeiss, Hertfordshire, UK). Periadventitial fibroadipose tissue and connective tissue were removed from the vessel and its aortic arch, whilst pinned out (Minutien Pins; FST, INTERfocus Ltd, Cambridge, UK) in a silicone-coated dissecting dish (Sylgard, Dow-Corning, UK) containing PSS (at RT). Clean aortae were either tissue-tap dried and frozen ( $-80^{\circ}\text{C}$ ) or processed for functional analysis (sections 2.4.5.1 and 2.4.5.2).

##### **2.4.5.1. Frozen Aortae**

Whole, frozen aortae were pulverised in chilled tin foil under liquid nitrogen, using mortar and pestle, and then mechanically homogenised in ice-cold Krebs or Phosphate buffer (0.5 ml). Homogenates were kept on ice at all times, the remaining solid tissue was deposited by centrifugation (4000 g,  $4^{\circ}\text{C}$ , 2 min) and the supernatant was removed. An aliquot (40  $\mu\text{l}$ ) of supernatant was retained for immediate determination of protein concentration (section 2.4.7). Remaining samples were stored at  $-80^{\circ}\text{C}$  until use.

#### **2.4.5.2. Fresh Aortae**

Freshly cleaned aorta (as described in section 2.4.5) was cut into 1.5 – 2 mm segments (4-5 rings from one animal) and immediately placed in either PSS buffer or DMEM medium without L-arginine, in a 24-well plate or mounted onto the myograph for incubation (section 2.10).

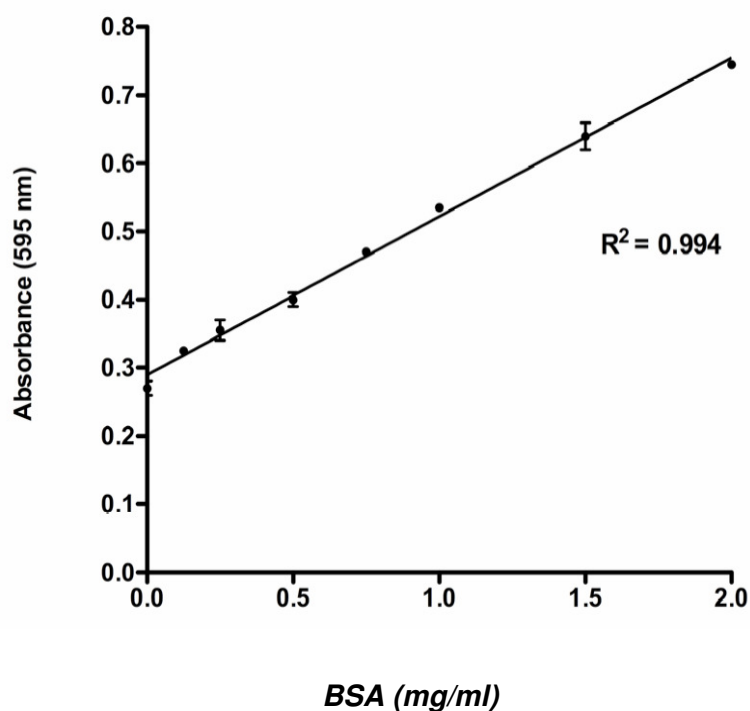
#### **2.4.6. Plasma Sampling**

Approximately 1ml of plasma was collected after pooling the blood from 2 – 3 mice. Blood was collected in EDTA-coated (1.6 mg/ml) vials (9 ml, Sarstedt, Monovette, Numbecht, Germany), gently inverted to mix pooled blood and plasma was separated by centrifugation (2000 g, 4°C, 15 min) and transferred to a fresh eppendorf. An aliquot of BHT antioxidant (50 µg/5µl, in ethanol) was added to each plasma sample (Sevanian *et al.*, 1994; Siems *et al.*, 2005; Tsai, 1984), argon was bubbled in and samples stored at –80°C.

#### **2.4.7. Quantitation Of Protein - Bradford Method**

The protein concentrations of tissue homogenates and subcellular fractions were determined colorimetrically using a Bio-Rad protein assay kit (Bio-Rad, Hemel Hempstead, UK) and the Bradford method of detection (Bradford, 1976). Protein assay dye reagent was diluted in HPLC water (1:5, freshly prepared) as well as a series of bovine serum albumin protein standards (concentration range 0.125 - 2 mg/ml, stored at –20°C) prepared in water. Water was used as the Blank. The assay was performed in duplicate, by adding Bio-Rad diluted dye (200 µl) to the protein sample and protein standards (each, 10 µl). The dilutions of unknown proteins were prepared to yield concentrations, which corresponded to the range of the standard curve, based on the initial assessment of the colour change upon mixing the protein and the diluted dye. The plate was shaken to mix and allowed to stand (RT, 15 min –

1h). UV absorbance was measured ( $\lambda 595$  nm) on a UV/visible spectrophotometer (Shimadzu, Milton Keynes, UK) and protein concentrations of undiluted samples were calculated (mg/ml) from the standard curve, allowing for the dilution factor. An example of a standard curve is shown in Figure 2.4 –1.



**Figure 2.4-1 Protein assay standard curve generated for the calculation of protein concentration.**

Standard curves for Bio-Rad protein assay were generated from UV absorbance ( $\lambda 595$  nm) of known concentrations of bovine serum albumin (BSA, 0.125 – 2 mg/ml) and used to determine the unknown concentration of samples by interpolating their UV absorbance against the standard curve.



## **2.5. Metabolism Of Steroids And Oxysterols By Murine Recombinant 11 $\beta$ HSD1 (m11 $\beta$ HSD1)**

### **2.5.1. Recombinant m11 $\beta$ HSD1 Protein Purification**

A protein pellet (15 g) containing m11 $\beta$ HSD1 was a gift from Dr Scott Webster (Endocrinology, University of Edinburgh). The cell pellet was re-suspended in “BugBuster” (Novagen, Merck Chemicals Ltd, Nottingham, UK) protein extraction reagent (5 ml/g of pellet), with lysonase (10  $\mu$ l/g pellet, Novagen); i.e. endonuclease for degradation of all forms of DNA and RNA in order to increase protein yield. For complete inhibition of serine and cysteine proteases, one crushed protease inhibitor cocktail tablet (Complete Mini, EDTA-free, Roche, Indianapolis, USA) was added and the mixture incubated at RT with shaking (50 rpm, 30 min), then separated (6000 g, 4°C, 20 min) and the supernatant collected.

Concomitantly, Talon Resin (Clontech, Saint-Germain-en-Laye, France) was prepared in five separate Falcon tubes (15 ml) by washing in ethanol (2x3ml), followed by centrifugation (6000 g, 4°C, 2 min), after which the ethanolic fractions were discarded. Talon resin was further washed with the extraction buffer (2x3ml, as listed in section 2.1.2.) and buffer was discarded leaving the activated resin. The supernatant was applied to the above resin, mixed and separated (6000 g, 4°C, 2 min) and the supernatant removed (this represented Fraction 1, 15 ml). Tris extraction buffer (3 ml) was added to the resin and the supernatant collected after centrifugation (Fraction 2, 15 ml). This step was repeated generating Fraction 3 (15 ml). Imidazole (3 ml, 10 mM) was then applied (3x) and all centrifugal supernatants saved (fractions 4, 5 and 6, 15 ml each). Imidazole solutions (3 ml of 100 mM and 500 mM) were then added sequentially to the resin, generating Fractions 7 and 8 (15 ml each), following centrifugation.

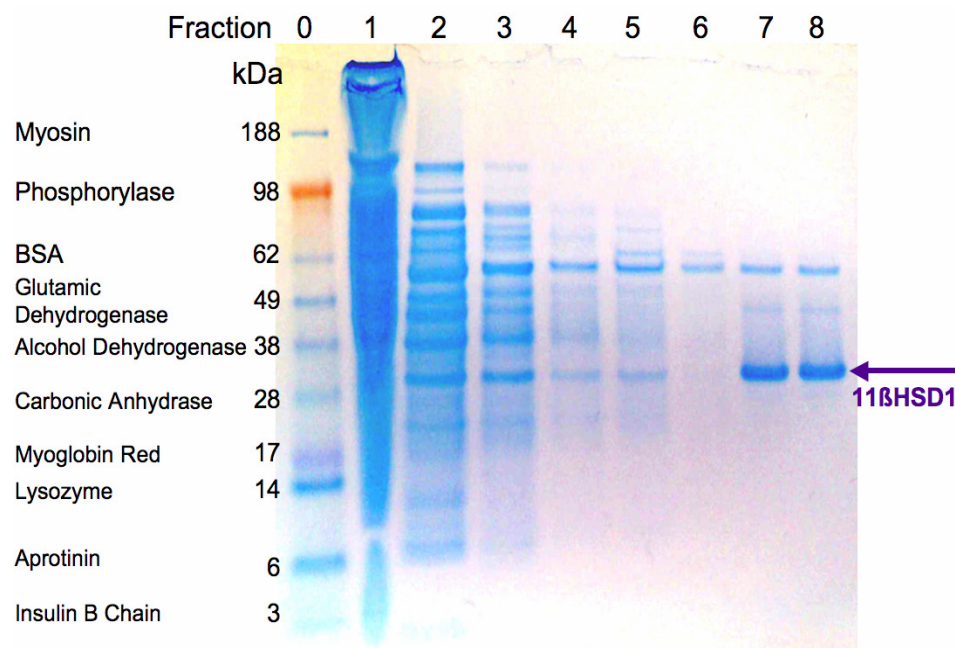
Samples were prepared for electrophoresis from each of the above fractions containing: protein fraction (10  $\mu$ l), NusPage sample buffer (4  $\mu$ l) and protein reducing agent (1  $\mu$ l), (Paisley, Invitrogen, UK). Samples were heated (100°C, 5

min) and analysed by electrophoresis (200V, 400A, 30min), using Bis-Tris gel (12%, w/v, Invitrogen) with NusPage MES SDS (20x) running buffer along with protein standards (4 – 250 kDa, SeeBlue Plus-2 Pre-stained standard, Invitrogen). The gel was washed in water (3x) before Simply Blue Safe Stain (containing Coomassie G-250, Invitrogen) was added (1 – 3h) to visualize the proteins on a gel. After staining, the gel was washed in water (2x) and a 32 kDa band, corresponding to m11 $\beta$ HSD type 1 was observed with Fraction 7 and Fraction 8, as anticipated (Figure 2.5 – 1). These two fractions with m11 $\beta$ HSD1 protein were further purified by dialysis using two separate dialysis tubes (0.05  $\mu$ m, Milipore, Watford, UK) placed in extraction buffer (5L, 4°C, stirred, overnight).

The two fractions containing m11 $\beta$ HSD1 were combined (30 ml) and protein concentration was determined (section 2.4.7). Total protein (30 ml) was further concentrated (to 2 ml), using a Vivaspın20 10.000 kDa centrifugal concentrator (2x20 ml, VivaScienceAG, Hanover, Germany) by continuous spinning (3000 g, 4°C, 30 – 90 min). The resultant purified m11 $\beta$ HSD1 protein was present at a concentration of 1.4 mg/ml (2 ml) and was stored in aliquots (100 – 500  $\mu$ l, 4% glycerol) at –20°C.

### **2.5.2. Oxidation Of Corticosterone To 11-Dehydrocorticosterone**

Corticosterone (B, 10 mM, in ethanol) was stored at –20°C, following sonication, and further ethanolic dilutions of B were prepared as required. Recombinant m11 $\beta$ HSD1 protein (28  $\mu$ g/ml) was incubated with unlabelled B (0.02 – 40  $\mu$ M) and NADP (2 mM) in Krebs Buffer (250  $\mu$ l, final volume). Samples were incubated (0 – 120 min) and the reaction stopped by freezing (–20°C) or adding ethyl acetate (10x volume). The internal standard, epi-F (10  $\mu$ g/ml, in ethanol) was added and steroids further extracted (3x) into ethyl acetate and the organic phase reduced to dryness under a stream of nitrogen (OFN, 60°C) and dried residues were stored at –20°C until subsequent analysis by HPLC–UV (section 2.12.1.2).



**Figure 2.5-1 Separation of m11 $\beta$ HSD1-purification fractions.**

Coumassie blue-stained SDS polyacrilamide gel (12%, NusPage) with fractions 1 – 8 collected upon purification of m11 $\beta$ HSD1 protein. Fractions 7 and 8 (eluted with 100mM and 500 mM Imidazole, respectively) contained 32 kDa m11 $\beta$ HSD1 protein. All bands were compared with protein standards in lane 0 (SeeBlue Plus2, 4–250 kDa) as indicated.

### 2.5.3. Oxidation Of 7 $\beta$ -Hydroxycholesterol To 7-Ketocholesterol

An ethanolic solution of 7 $\beta$ OHC (10 mM, containing 50  $\mu$ g/ml BHT) and subsequent ethanolic dilutions, were prepared as required and stored under argon at -20°C. The dehydrogenation of 7 $\beta$ OHC (1 – 20  $\mu$ M) was performed as described for steroids (section 2.5.2) with some modifications. The reaction was carried out using Phosphate Buffer (250  $\mu$ l, final volume) containing cysteamine-HCl (20 mM) (Song, et al., 1998), as it was initially observed that the reaction occurred at a faster rate in this solution than in Krebs. Internal standards, C4 (5  $\mu$ g/ml) and 19OHC (10  $\mu$ g/ml), were added in place of epi-F, after the incubation. Oxysterol extraction from Phosphate Buffer was performed using petroleum ether (3x 6 ml). Organic extracts were reduced to dryness under argon (RT) and stored at -20°C until analysis by HPLC–UV (section 2.12.1.3).

### 2.5.4. Reduction Of 11-Dehydrocorticosterone To Corticosterone

#### 2.5.4.1. *Preparation Of Cofactor (NADPH) Regenerating System*

NADPH (20 mM) was prepared by co-incubating NADP<sup>+</sup> (20 mM), glucose-6-phosphate (40 mM) and glucose-6-phosphate dehydrogenase (0.1 unit/ml, from yeast, Roche, Mannheim, Germany). The enzyme was dissolved and stored in aliquots (0.1 unit/10  $\mu$ l, sodium phosphate buffer containing 8% glycerol, as in section 2.2) at -20°C. NADPH generating mixture was mixed, covered in tin foil and stored on wet ice until use on the same day. Before use in the assay, the mixture was incubated (10 min, 37°C).

#### 2.5.4.2. *Assay Conditions*

Ethanolic dilutions of unlabelled 'A' were prepared from concentrated stock (6 mM, in ethanol) on the day, to achieve the required concentration. The reaction mixture contained: NADPH generating system (2 mM, final volume, see section 2.5.4.1), m11 $\beta$ HSD1 protein (28  $\mu$ g/ml), unlabelled A (0.02 – 40  $\mu$ M) in Krebs buffer (250  $\mu$ l, final volume). Internal standard (epi-F, 10  $\mu$ g/ml) was added following

incubation (15 min – 3h), and steroids extracted exactly as described (section 2.5.2) until analysis by HPLC–UV (section 2.12.1.2).

### **2.5.5. Reduction Of 7-Ketocholesterol To 7 $\beta$ -Hydroxycholesterol**

An ethanolic solution of 7-KC (10 mM, containing 50  $\mu$ g/ml BHT) and subsequent ethanolic dilutions were prepared and stored under argon at -20°C. The reductase assay was prepared as described (in section 2.5.3) for the dehydrogenase reaction, with minor amendments. m11 $\beta$ HSD1 protein (28  $\mu$ g/ml) was incubated with 7-KC (0.5 – 40  $\mu$ M) and the NADPH generating system (2 mM, as in section 2.5.3.1) in phosphate buffer (250  $\mu$ l, final volume). Samples were further incubated, oxysterols extracted and stored as described in section 2.5.3 until analysis by HPLC–UV (section 2.12.1.3).

### **2.5.6. Controls And Quantitation**

In each of the above assays, control samples were included: “Blank” (with substrate, cofactor, but no protein), “No-cofactor” (with substrate and protein but no cofactor) and ‘Just protein’ (containing buffer and protein only). Tubes were maintained on wet ice, and substrate and cofactor were added last in quick succession. In all assays reactions were performed at least in duplicate and time of incubation was established for each reaction to allow product formation to conform to first order linear kinetics (approximately up to 30% conversion).

With each assay, two duplicate standard curves were constructed using a series of dilutions representing known concentrations of A and B (0.02 – 40  $\mu$ M) or 7-KC and 7 $\beta$ OHC (0.04 – 40  $\mu$ M), as appropriate, and a fixed amount of relevant internal standard (as given above). The samples for standard curve preparation also contained cofactor (2 mM) and appropriate buffer (250  $\mu$ l, final volume), but lacked protein, and were incubated simultaneously with the assay. Typically a minimum of eight points, in duplicate, were used on a standard curve.

## **2.6. Metabolism Of Steroids And Oxysterols By m11 $\beta$ HSD1 Stably Transfected In CHO and HEK Cells**

### **2.6.1. Cell Culture**

All work was carried out in a tissue-culture hood and employed general sterile tissue-culture laboratory techniques. Both HEK (Human embryonic kidney) and CHO (Chinese hamster ovary) cell types (from the European Collection of Cell Cultures EACC, Porton Down, UK 05030204 and 85050302 respectively), stably transfected with mouse or human 11 $\beta$ HSD1 or 11 $\beta$ HSD2, were a generous gifts from Dr Scott Webster and Mrs Margaret Binnie (Endocrinology, Edinburgh University).

#### **2.6.1.1. *Charcoal-Stripped Serum***

Foetal calf serum (FCS, 0.5L) was defrosted at RT and dextran-coated charcoal (5 g) was added and stirred overnight (4°C). The mix was subjected to double filtration through sterile filter units (0.45  $\mu$ m, then 0.22  $\mu$ m, 500 ml volume), then heat-inactivated (56°C, 30 min in water bath), cooled to RT, separated in aliquots (50 ml) and stored at -20°C.

#### **2.6.1.2. *Thawing And Passaging Cells***

A cryovial of frozen cells, which had been stored in liquid nitrogen (-196°C), was placed on dry ice. Cells were thawed very quickly by agitating the vial by hand at 37°C in a water bath, and then transferred to a T75 cm<sup>2</sup> flask containing 10 ml pre-warmed medium.

Cells were maintained on a T75 cm<sup>2</sup> dish in a humidified atmosphere (5% CO<sub>2</sub>, 95% air, 37 °C) in an incubator for 48 h. HEK cells were maintained in Dulbecco's modified Eagle's medium (DMEM) and seeded on poly-D-lysine coated plates (50  $\mu$ g/ml, 5 min), whereas for CHO cells, Ham's F12 essential medium (Ham's F12) was used. Both media were supplemented with glutamine (2 mM), penicillin (100 units/ml), streptomycin (100  $\mu$ g/ml), and heat-inactivated foetal calf serum

(FCS, 10% v/v). For CHO cells transfected with mouse or human 11 $\beta$ HSD1, Ham's F12 medium was further supplemented with 100  $\mu$ g/ml hygromycine.

Prior to passaging, the cells were washed in PBS without calcium and magnesium (5 – 10 mls, to remove divalent cations and serum). The cells were enzymatically displaced by adding 1x trypsin-EDTA (50 units/ml trypsin and 5 mM EDTA) solution (2 ml for T75 cm<sup>2</sup> dish) and placed in an incubator (37°C, 2 – 5 min) until the cells had detached. Detachment of cells was carefully monitored under a light microscope. Often the flask was gently tapped in order to loosen all the cells. Cell number was counted using a haemocytometer. Trypsin was inactivated by adding fresh medium containing 10% FCS (15 ml in T75 cm<sup>2</sup>). The cell suspension was further split (1:5) between new T75 cm<sup>2</sup> flasks, containing fresh pre-warmed medium. The flasks were returned to an incubator for cells to adhere until they were confluent (90%). Medium was changed every 2 – 3 days by aspirating and was discharged and replaced with a fresh pre-warmed medium.

Prior to performing steroid or oxysterol assays, appropriate cells were seeded and treated with medium as above, containing dextran-coated charcoal stripped-FCS (10%, v/v) instead of FCS. When cells were confluent (90%), they were further seeded onto 6-well plates (PDL-coated, where appropriate) and maintained in medium with stripped serum to attach overnight. The same medium with stripped serum was used subsequently in all assays.

Cell viability in tissue culture was measured by trypan blue exclusion staining. Counting was performed in triplicate and results averaged.

#### **2.6.1.3. Freezing And Storage Of Cells**

For long-term storage, 80 - 95% confluent cells were detached with trypsin/EDTA as described in section 2.6.1.2. Cells were counted using a haemocytometer. The remainder of the cell suspension was subjected to centrifugation (210 g, RT, 5 min) and the resultant pellet was re-suspended at 2x10<sup>6</sup> cells/ml in FCS containing dimethylsulphoxide (DMSO, 10% v/v). The cell content was transferred to a cryovial

and placed inside a polystyrene rack, which was transferred onto dry ice for slow freezing, then placed at  $-80^{\circ}\text{C}$ . After several days, the vials were transferred to liquid nitrogen for long-term storage. This freezing procedure ensures gradual freezing of the cells allowing better preservation, and improved success when reviving them from the low temperature.

### **2.6.2. Oxidation Of Corticosterone To 11-Dehydrocorticosterone**

Aliquots of radio-labelled substrate ( $[^3\text{H}]_4\text{-B}$ ) and unlabelled B were mixed and reduced to dryness (OFN,  $60^{\circ}\text{C}$ ). The residue was dissolved in ethanol (0.01%, v/v) in DMEM to achieve concentrations of  $[^3\text{H}]_4\text{-B}$  (5 nM) and B (25 nM) and this medium was applied to the cells for 90 min. Steroids were then extracted from the medium as follows.

#### **2.6.2.1. Extraction Of Steroids From The Medium**

The method from Small *et al.*, (2005) was adapted to give >90% extraction efficiency. The extraction was performed using Sep-pak C18 columns (360 mg, 55-105  $\mu\text{m}$ , Waters, Bedford, UK), attached to an empty syringe (2 ml) and a vacuum chamber, and further prepared for extraction by washing with methanol (2 ml), then water (5 ml), under a mild vacuum. Medium was passed through the column and the steroids retained. The columns were washed with water (5 ml) and steroids were eluted with methanol (2 mls). The eluate volume was reduced to a half, under OFN ( $60^{\circ}\text{C}$ ) and steroids were further extracted into ethyl acetate (2x3 ml, 400 rpm, RT). The organic phase was reduced to dryness (OFN,  $60^{\circ}\text{C}$ ) and residues stored at  $-20^{\circ}\text{C}$  until analysis by HPLC with radiodetection (see section 2.12.2.1).

### **2.6.3. Oxidation Of $7\beta$ -Hydroxycholesterol To 7-Ketocholesterol**

Freshly made DMEM containing stripped-FCS (10%, v/v) was used to prepare test solutions of unlabelled  $7\beta\text{OHC}$  (1  $\mu\text{M}$ , 0.01% v/v ethanol). Cells were incubated (6 – 24 h,  $37^{\circ}\text{C}$ ) and oxysterols were extracted from the medium and cells as follows:



### **2.6.3.1. Extraction Of Oxysterols From The Medium**

Medium was removed from cells into a glass vial (20x1.5 cm) and a mixture of deuterium-labelled internal standards, d7-7-KC (2 µg/50 µl) and d7-7βOHC (0.5 µg/50 µl) was added in a single aliquot (50 µl). Argon gas was flushed through all samples, and oxysterols were extracted (3x3 ml, 400 rpm, RT, 15 min) from DMEM media with a mixture of hexane:2-propanol (60:40), and from Ham's F12 medium with chloroform:methanol (2:1). The organic phases were combined, evaporated under the stream of argon (RT) and residues dissolved in chloroform:methanol (2:1, 350 µl), then stored at -20°C until analysis by GC/MS (see section 2.13).

### **2.6.3.2. Extraction Of Oxysterols From The Cells**

After removing the medium, cells were washed in ice-cold PBS (twice, 2 ml/well) and lysed (200 µM, NaOH, 0.6 ml/well) by rocking the plate (60 rpm, 4°C, 20 min). The contents of each well were forced through a pipette tip repeatedly, for dispersal. An aliquot (100 µl) was removed for determination of protein concentration, and ice-cold PBS with EDTA (200 µM, 0.5 ml) was added. This step was proven necessary by Brown *et al.*, (2000b) as it increases extraction efficiency of 7-KC from cells by 30%. Internal standards were added as described for media (section 2.6.3.1) and oxysterols immediately extracted (400 rpm, RT, 2 min) from lysed cells into methanol (2.5 ml) and hexane (10 mls). Phase separation was achieved (1000 g, 4°C, 10 min) and the organic (upper) phase was removed and evaporated under argon (RT). The dried residues were dissolved in chloroform:methanol (2:1, 100 µl) and stored at -20°C until analysis by GC/MS (see section 2.13).

## **2.6.4. Reduction Of 11-Dehydrocorticosterone To Corticosterone**

### **2.6.4.1. Preparation Of [<sup>3</sup>H]<sub>4</sub> - 11-Dehydrocorticosterone**

[<sup>3</sup>H]<sub>4</sub>-11-dehydrocorticosterone (A) was always prepared from a freshly opened stock of [<sup>3</sup>H]<sub>4</sub>-corticosterone (B) in the following reaction mixture:

[ $^3\text{H}$ ] $_4$ -B (50  $\mu\text{l}$ , 4  $\mu\text{M}$ ),  $\text{NAD}^+$  (200  $\mu\text{l}$ , 25 mM) and placental homogenate (300  $\mu\text{l}$ , section 2.4.4) were mixed in 'C' buffer (5 ml, final volume). The reaction was prepared in glass tubes, split between 3 tubes (~1.7 ml each) and incubated in a water bath with gentle rocking (60 rpm, 37°C, 4 h). Steroids were extracted into ethyl acetate (3x5 ml), by vortexing (400 rpm, RT, 2 min), and upper phases were combined and reduced to dryness (OFN, 60°C). The dry residue was dissolved in toluene (300  $\mu\text{l}$ ). To assess whether the conversion of [ $^3\text{H}$ ] $_4$ -B  $\rightarrow$  [ $^3\text{H}$ ] $_4$ -A was complete, an aliquot of product was analysed by HPLC (as in section 2.12). The concentration of [ $^3\text{H}$ ] $_4$ -A was calculated by measuring cpm/ $\mu\text{l}$  of radioactive emissions and comparing with that of initial [ $^3\text{H}$ ] $_4$ -B of known concentration. Cpm/ $\mu\text{l}$  were assessed by scintillation counting in the presence of scintillation fluid (3 ml, Meridian, Surrey, UK).

#### **2.6.4.2. Incubation With [ $^3\text{H}$ ] $_4$ - 11-Dehydrocorticosterone**

Radio-labelled substrate ([ $^3\text{H}$ ] $_4$ -A, 5 nM, prepared as in section 2.6.4.1) together with unlabelled A (25 nM) was added to cells in medium exactly as described for the dehydrogenase reaction (2.6.4), with the exception of shorter incubation time (60 min).

#### **2.6.5. Reduction Of 7-Ketocholesterol To 7 $\beta$ -Hydroxycholesterol**

Cells were treated with 7-KC (1  $\mu\text{M}$ , 0.01% v/v, ethanol) instead of [ $^3\text{H}$ ] $_4$ -A and A exactly as given in section 2.6.4.

#### **2.6.6. Controls And Quantitation**

Individual experiments were performed using cells from four separate passages. As a control, CHO and HEK cells not transfected with m11 $\beta$ HSD1 were also used in order to account for residual levels of steroids or oxysterols in these cells. Cells transfected with m11 $\beta$ HSD1 protein were also treated with medium containing ethanol (0.01%) as a vehicle, in place of [ $^3\text{H}$ ] $_4$ -steroid or 7-oxysterol, and a control with medium alone without cells was also included. One 6-well plate typically contained 7-KC, 7 $\beta$ OHC and ethanol treatment (2 ml/well) or [ $^3\text{H}$ ] $_4$ -A, [ $^3\text{H}$ ] $_4$ -B and

ethanol treatment (2 ml/well). Treatments were performed in duplicate. For quantitation of oxysterols, standard curve samples were incubated on a 24-well plate, in duplicate. Medium (1 ml) with known concentrations of both 7 $\beta$ OHC and 7-KC (5, 25, 50, 125, 250, 500 and 1000 nM each) were incubated together and separately (1000 nM, 7-KC or 7 $\beta$ OHC only) for the duration of treatment. A new standard curve was generated during each assay to control for subtle differences in incubation and extraction of oxysterols from the medium.

### **2.6.7. Metabolism Of Oxysterols By 11 $\beta$ HSD1 In Cell Lysates**

HEK cells and CHO cells both stably transfected with m11 $\beta$ HSD1 were used to prepare cell lysates.

Cells were washed in ice-cold PBS, scraped off T75 cm<sup>2</sup> flask with a plastic scraper and 'TG1' lysis buffer (2 ml) was added. Cells were lysed by rocking the plate (60 rpm, 4°C, 20 min). Supernatant from the lysed cells was collected after centrifugation (4,000 g, 4°C, 10 min) and stored in aliquots (500  $\mu$ l) at -80°C. A further aliquot (50  $\mu$ l) was removed for determination of protein concentration (Section 2.4.7).

#### **2.6.7.1. Reduction Of 7-Ketocholesterol To 7 $\beta$ -Hydroxycholesterol**

The assay of 7-KC conversion to 7 $\beta$ OHC was performed as described (section 2.5.5) for recombinant protein, with minor modifications. Cell lysates (50-100  $\mu$ g/ml) were utilised with unlabelled 7-KC (500 nM - 5  $\mu$ M) and radio-labelled substrate [<sup>3</sup>H]<sub>3</sub>-7-KC (5 nM) and the reaction was incubated for 30 min.

#### **2.6.7.2. Controls And Quantitation For Cell Lysates**

The 'Blank', 'No-cofactor', 'Just protein' and un-labelled substrate controls were included per assay as described for murine recombinant protein (section 2.5.6). In addition, radiolabelled substrate, [<sup>3</sup>H]<sub>4</sub>-A, was used at a single concentration (5 nM) as a control for chemical degradation in the assay.

## **2.7. Metabolism Of Steroids And Oxysterols By Microsomal 11 $\beta$ HSD1 Protein**

Hepatic microsomes were prepared as described in section 2.4.2.

### **2.7.1. Oxidation Of Corticosterone To 11-Dehydrocorticosterone**

The 11 $\beta$ HSD1 dehydrogenase reaction (B $\rightarrow$ A) mixture included: [ $^3$ H] $_4$ -B (5 nM), unlabelled B (20 - 1000 nM), mouse hepatic microsomes (200 – 400  $\mu$ g/ml) and NADP $^+$  (2 mM), incubated in Krebs buffer (250  $\mu$ l, final volume, 37°C, 30 min). The reaction was stopped by freezing (-20°C) or adding ethyl acetate (10x volume) into which steroids were extracted and the organic phase reduced to dryness (OFN, 60°C). Dried residues were stored at -20°C until analysis by HPLC –radiodetection as described (section 2.12.2.1).

### **2.7.2. Oxidation Of 7 $\beta$ -Hydroxycholesterol To 7-Ketocholesterol**

The dehydrogenation of 7 $\beta$ OHC to 7-KC was performed in phosphate buffer rather than Krebs. The reaction mixture contained: 7 $\beta$ OHC (0.02 - 20  $\mu$ M), mouse hepatic microsomes (200 - 400  $\mu$ g/ml) and NADP $^+$  (2 mM), incubated in phosphate buffer (250  $\mu$ l, final volume, 37°C, 30 min-1h). The reaction was stopped by freezing (-20°C) or adding petroleum ether (10x v/v) then internal standards C4 (5  $\mu$ g/ml) and 19OHC (10  $\mu$ g/ml) were added and oxysterols extracted (3x, 400 rpm, RT, 2min). Collated extracts were evaporated under argon and residues were stored at -20°C until analysis by HPLC – UV (section 2.12.1.2).

### **2.7.3. Reduction Of 11-Dehydrocorticosterone To Corticosterone**

The reductase assays with mouse hepatic microsomes were set up as described for dehydrogenase (section 2.7.1) using [ $^3$ H] $_4$ -A substrate (5 nM) and unlabelled A (20 – 1000 nM), in place of [ $^3$ H] $_4$ -B, and B; and the NADPH cofactor generating system (2 mM, section 2.5.4.1) instead of NADP $^+$ . Samples were processed as described in

section 2.7.1 and stored at -20°C until analysis by HPLC – radiodetection (section 2.12.2.1).

#### **2.7.4. Reduction Of 7-Ketocholesterol To 7 $\beta$ -Hydroxycholesterol**

The reductase assay with mouse hepatic microsomes was set up as in section 2.7.2, but using 7-KC rather than 7 $\beta$ OHC as the substrate and the NADPH cofactor generating system (2 mM, section 2.5.4.1) instead of NADP<sup>+</sup>.

#### **2.7.5. Controls And Quantitation**

In all microsomal dehydrogenase assays control samples of ‘Blank’, ‘No-cofactor’ and ‘Just protein’ were included and standard curve prepared as described for mouse recombinant protein in section 2.5.6.

### **2.8. Metabolism Of Steroid And Oxysterols By 11 $\beta$ HSD1 In Aortae**

For measurement of 11 $\beta$ HSD1 activity in the vessel wall, aortic rings were prepared as described (section 2.4.5.2). All assays were performed on a 24-well plate by incubating the aortic rings (one/well, 37°C, 95% CO<sub>2</sub>, 5% O<sub>2</sub>) in DMEM (containing supplements as described in section 2.6.2) with [<sup>3</sup>H]<sub>4</sub>-steroids or oxysterols, typically for 16 - 24 hours. The order in which the rings were utilised was standardized and segments were dissected from the aortic arch then immersed in DMEM containing EtOH (vehicle), 7-KC, 7 $\beta$ OHC, [<sup>3</sup>H]<sub>4</sub>-A and [<sup>3</sup>H]<sub>4</sub>-B.

#### **2.8.1. Oxidation Of Corticosterone To 11-Dehydrocorticosterone**

DMEM containing [<sup>3</sup>H]<sub>4</sub>-B (10 nM) was prepared as in section 2.6.3, and was used to measure dehydrogenation by aortic rings, as reported previously, Small *et al.*, (2005). The extraction and detection of steroids was performed as described for cultured cells (section 2.6.3.1). Dried residues were stored at -20°C until analysis by HPLC – radiodetection (section 2.12.2.1).

### **2.8.2. Oxidation Of 7 $\beta$ -Hydroxycholesterol To 7-Ketocholesterol**

Aortic rings were incubated in DMEM containing 7 $\beta$ OHC (1  $\mu$ M) exactly as described for cells (section 2.6.3).

### **2.8.3. Reduction Of 11-Dehydrocorticosterone To Corticosterone**

DMEM containing [ $^3$ H] $_4$ -A (10 nM) in place of [ $^3$ H] $_4$ -B was added to aortic rings as in section 2.8.1.

### **2.8.4. Reduction Of 7-Ketocholesterol To 7 $\beta$ -Hydroxycholesterol**

Aortic rings were incubated in DMEM containing 7-KC (1  $\mu$ M) as described for cultured cells (section 2.6.5).

### **2.8.5. Controls And Quantitation**

All assays included vehicle controls (medium containing ethanol (0.01%, v/v) in place of [ $^3$ H] $_4$ -steroids or 7-oxysterol), negative controls for tissues (medium and steroid or oxysterol without aortic rings) and blank samples (medium alone). For quantitation of oxysterols, standard curves were prepared as described in section 2.6.7. After incubation, aortic rings were removed from each well, blotted on tissue paper and weight was recorded for subsequent velocity calculations (pmol/mg/day).

## **2.9. Metabolism Of Steroids And Oxysterols By 11 $\beta$ HSD2**

### **2.9.1. h11 $\beta$ HSD2 Stably Transfected In CHO Cells**

CHO cells stably transfected with human 11 $\beta$ HSD2 (h11 $\beta$ HSD2) protein were maintained as in section 2.6.1 and used in subsequent steroid and oxysterol reaction assays.

#### ***2.9.1.1. Oxidation Of 7 $\beta$ -/7 $\alpha$ -Hydroxycholesterol To 7-Keto-cholesterol***

Assessment of dehydrogenation of 7 $\beta$ - or 7 $\alpha$ - hydroxycholesterols (1  $\mu$ M) by 11 $\beta$ HSD2 in CHO cells was performed as described for m11 $\beta$ HSD1 in HEK cells (section 2.6.3). The deuterium-labelled internal standards (d7-7-KC, 2  $\mu$ g/50  $\mu$ l and d7-7 $\beta$ OHC, 0.5  $\mu$ g/50  $\mu$ l) were added and samples of cells and medium were processed as in sections 2.6.3.1 and 2.6.3.2. Dried organic extracts of oxysterols were stored at -20°C for subsequent analysis by GC/MS (section 2.13).

#### ***2.9.1.2. Reduction Of 7-Ketocholesterol To 7 $\beta$ -Hydroxy-cholesterol***

The reduction of 7-KC (1  $\mu$ M) by h11 $\beta$ HSD2 in intact CHO cells was performed as described for m11 $\beta$ HSD1 in HEK cells (section 2.6.5). Dried organic extracts of oxysterols were stored at -20°C for subsequent analysis by GC/MS (section 2.13).

#### ***2.9.1.3. Controls And Quantitation***

In all assays controls were included and samples processed to generate a standard curve, as in section 2.6.6.

### **2.9.2. Renal Homogenates**

Homogenates of mouse kidneys were prepared as described (section 2.4.3).

### **2.9.2.1. Oxidation Of Dexamethasone To 11-Dehydro-dexamethasone**

Dexamethasone (DEX, 1 mM, in ethanol) was stored at -20°C, following sonication, and ethanolic dilutions were prepared as required. Renal homogenates as a source of m11 $\beta$ HSD2 protein (200 – 400  $\mu$ g/ml) were incubated with DEX (40  $\mu$ M) and NAD<sup>+</sup> (2 mM) in Krebs Buffer (250  $\mu$ l, final volume). The internal standard (epi-F, 10  $\mu$ g/ml) was added after stopping the reaction. Samples were further extracted (section 2.5.2) into ethyl acetate (10x volume) and dried residues processed for analysis by HPLC – UV (section 2.12.1.1).

### **2.9.2.2. Oxidation Of 7 $\beta$ -Hydroxycholesterol To 7-Keto-cholesterol**

The ability of renal m11 $\beta$ HSD2 to utilize 7 $\beta$ OHC (1 – 20  $\mu$ M) as a substrate was tested as described for DEX in section 2.9.2.1 but the reactions were performed in Phosphate Buffer. Internal standards d7-7-KC (2  $\mu$ g/50  $\mu$ l) and d7-7 $\beta$ OHC (0.5  $\mu$ g/50  $\mu$ l) were added after stopping the reaction. Samples were extracted as described for oxysterols with m11 $\beta$ HSD1 (section 2.5.3), and evaporated residues stored at -20°C until subsequent analysis by GC/MS (section 2.13).

### **2.9.2.3. Reduction Of 11-Dehydrodexamethasone To Dexamethasone**

Dilutions of 11-dehydrodexamethasone (11-DHDEX) were prepared from stock (1 mM, in ethanol) as required. The reductase assay mixture was as described (section 2.9.2.1) except for 11-DHDEX (40  $\mu$ M) and NADH (2 mM) cofactor used in place of DEX and NAD<sup>+</sup>. Samples were further extracted as described in section 2.9.2.1 and processed for analysis by HPLC – UV (section 2.12.1.1).



#### **2.9.2.4. Reduction Of 7-Ketocholesterol To 7 $\beta$ -Hydroxy-cholesterol**

Reduction of 7-KC (1  $\mu$ M – 20  $\mu$ M) by m11 $\beta$ HSD2 substrate was assessed in phosphate buffer instead of Krebs, as described (section 2.9.2.3). Internal standards d7-7-KC (2  $\mu$ g/50  $\mu$ l) and d7-7 $\beta$ OHC (0.5  $\mu$ g/50  $\mu$ l) were added after stopping the reaction. Samples were processed for extraction as described (section 2.5.5) and evaporated residues stored at –20°C for analysis by GC/MS (section 2.13).

#### **2.9.2.5. Controls And Quantitation**

Control samples were included and standard curves prepared for subsequent quantitation of steroids and oxysterols exactly as described (sections 2.5.6 and 2.6.6).

### **2.10. Functional Studies In Isolated Mouse Aortae**

Small vessel wire myography (JP Trading, Denmark) was used to study isolated aortic ring preparations in order to measure responses to pharmacological agents. The response, as isometric force (mN), was measured in a Multi-Tissue wire myograph system 700MO (Danish Myo Technology, Copenhagen, Denmark) with four parallel chambers (6 ml) and data captured using MabLab/4e analogue-digital converter and displayed through Chart<sup>TM</sup> v3.6/s software (AD Instruments, Sussex, UK).

#### **2.10.1. Suspending Vessels In The Myograph**

Intact aortae were prepared for myography as described (section 2.4.5). Arterial rings (1.5 – 2 mm) were suspended between two parallel, intra-luminal wires, which were tightly secured onto a myograph (Mulvany and Halpern, 1976). Care was taken to prevent damaging the endothelium when inserting the wires (for de-endothelialisation see section 2.10.2). Rings were suspended in four parallel chambers (6 ml, one ring per chamber) containing oxygenated (95% O<sub>2</sub>, 5% CO<sub>2</sub>) PSS (37°C) at all times.

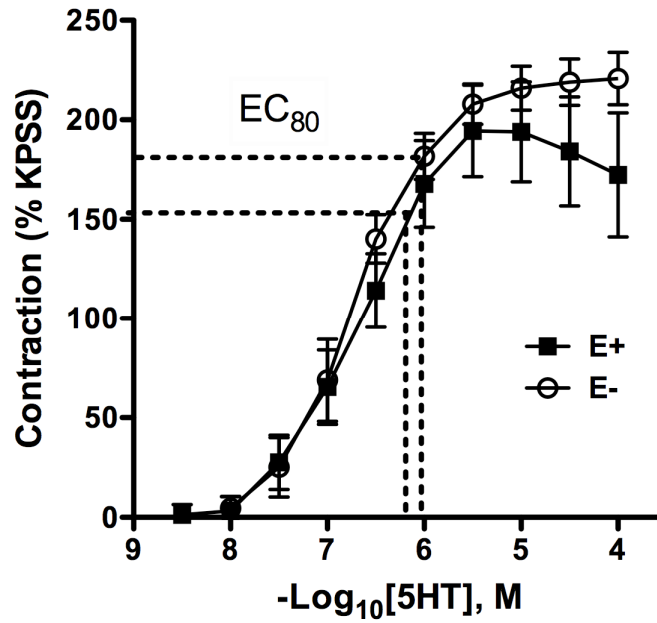
### 2.10.2. De-Endothelialisation

The most common method for removing the endothelium from small arteries is by mechanical disruption, usually by rubbing the luminal surface with a wire. Once the aortic rings were suspended in the myograph chamber, vessels were slightly stretched and a stainless steel Minutien pin (external diameter 150  $\mu\text{m}$ ; FST, Interfocus) was advanced into the lumen and used to gently rub the endothelial surface, taking care to minimize damage to the adjacent smooth muscle cells.

### 2.10.3. General Experimental Protocol

After mounting, the aortic rings were equilibrated in oxygenated buffer (95%  $\text{O}_2$  and 5%  $\text{CO}_2$ , 37°C), stretched to their optimum resting force (7.36 mN) (Bagnall *et al.*, 2006) and allowed to equilibrate (37°C, 20 – 30 minutes). As a standard initial procedure, maximum receptor-independent constriction in response to potassium ( $\text{K}^+$ ) was obtained by contracting the vessels three times with high potassium (125 mM) buffer (KPSS). Subsequently, cumulative concentration-response curves were obtained in each vessel for 5HT ( $10^{-9}$  –  $10^{-4}$  M) and a suitable concentration selected to produce 80% of the maximum response ( $\text{EC}_{80}$ ; 5HT,  $3 \times 10^{-7}$  M, Figure 2.10–1). Subsequently, concentration-response curves were generated for acetylcholine (ACh,  $10^{-9}$  –  $10^{-4}$  M), sodium nitroprusside (SNP,  $10^{-10}$  –  $10^{-3}$  M), noradrenaline (NA,  $10^{-9}$  –  $10^{-4}$  M) and KPSS. Responses to vasodilators were obtained following sub-maximal contraction ( $\text{EC}_{80}$ ) with 5HT (Figure 2.10-1).

In all experiments four aortic rings were used from each mouse; two with the endothelium undisturbed and a two from which the endothelium had been removed. Denudation was confirmed by the lack of relaxation to ACh following contraction with 5HT. The effect of 7-oxysterols on arterial function was further assessed by incubating the aortic rings with 7-KC or 7 $\beta$ OHC in tissue culture medium. All contractions were expressed as force (mN) and as a percentage of the maximum contraction in response to KPSS; relaxations were expressed as a percentage of the contraction in response to  $\text{EC}_{80}$  5HT. Data were expressed as the average of two aortic rings for each animal.



**Figure 2.10-1 Determining the EC<sub>80</sub> for 5HT in endothelium intact and denuded aortae.**

Cumulative concentration response curves to 5-hydroxytryptamine (5HT). Isometric force in response to 5HT ( $10^{-9}$  –  $10^{-4}$  M) was measured in isolated mouse aortic rings with denuded (E<sup>-</sup>) or intact (E<sup>+</sup>) endothelium (E). All contractions were expressed as a percentage of the maximum response to KPSS. The concentration of 5HT giving 80% of the maximum contraction was selected (EC<sub>80</sub>5HT,  $3 \times 10^{-7}$  M). All data are mean  $\pm$  SEM, n=8, in duplicate.

## 2.11. Extraction Of Oxysterols From Biological Fluids And Tissue

The detailed description of method development for processing of plasma and biological fluids is given in section 6.5.1. Internal standards were added to all samples prior to extraction (section 2.11.1). After each step of extraction, the dried residue was stored under argon at  $-80^{\circ}\text{C}$  until further processing for GC/MS.

### 2.11.1. Extraction From Mouse Plasma

#### a) Solvent extraction

All solvent mixtures (section 2.2.2) and mouse plasma samples (300 – 500  $\mu\text{l}$ ; section 2.4.6) were prepared as described. Argon was flushed through all samples and internal d7 standards were added (40 ng d7-7-KC, 10 ng d7-7 $\beta$ OHC and 1  $\mu\text{g}$  d7-cholesterol; total volume 50  $\mu\text{l}$ ). Oxidized cholesterol were extracted (twice) into diethyl ether in hexane (3 ml, 2:3). Samples were placed under argon, sealed and mixed vigorously (400 rpm,  $4^{\circ}\text{C}$ , 10 min). This step was repeated with each extraction. Organic phases were separated (1000 g,  $4^{\circ}\text{C}$ , 10 min), collated and evaporated under argon (RT). Once dissolved in toluene:ethyl acetate (1 ml, 1:1 mix), samples were processed through a solid phase extraction (SPE).

#### b) Solid-phase extraction (SPE)

Samples were prepared for gas-chromatography using solid phase extraction (Simpson *et al.*, (1992), Tippens *et al.*, (1988)). Samples (1ml) were applied to “Diol” columns (2OH, Bond Elute, Varian, 100mg, 1ml), which were pre-conditioned with toluene:ethyl acetate (2 ml, 1:1 mix). Oxidized cholesterol were eluted from the column with the same mix (2 ml) followed by chloroform (1ml). Total eluate (4 ml) containing cholesterol and cholesterol oxides was collected under mild vacuum. The samples were dried (argon, RT), dissolved in diethyl ether (2 ml) and subjected to hydrolysis.

*c) Hydrolysis (Saponification)*

In order to avoid decomposition of cholesterol and cholesterol oxides a modification of the methods of Brown *et al.*, (1997) and Hodis *et al.*, (1991) were applied involving mild ice-cold saponification, by incubating samples (60 rpm, 4°C, 2 h) in the dark (Tsai, 1984). In brief, samples were dissolved in diethyl ether (2 ml), and methanolic KOH (0.5 ml, 20% w/v) was added. Samples were sealed under argon, then covered in tin foil and mixed (60 rpm, 4°C, 2h).

*d) 2<sup>nd</sup> Solvent extraction*

Upon hydrolysis, the mixture was neutralized (pH 7.4) with acetic acid (300 µl, 20% v/v). Water (0.5 ml) and NaCl (0.15 M, 0.5 ml) were added to each sample to allow phase separation. Oxidized lipids were obtained following double extraction into the upper diethyl ether layer (400 rpm, RT, 2 min). Upon centrifugation (1000 g, 4 °C, 5 min) upper phases were collated and dried (argon, RT), then stored at -20°C until further analysis by GC/MS (Section 2.13.2).

It has been suggested by Csallany *et al.*, (1989) and shown by Brown *et al.*, (1997), that the analytical stages described should be performed rapidly, at low temperature, and in the absence of light (hydrolysis) and oxygen (by using argon gas), since the mere act of analysis may contribute to artefact formation during sample processing.

**2.11.2. Extraction From Tissues**

Aortic tissue (section 2.4.5.1) was homogenized under a stream of argon in chloroform:methanol (2:1 v/v) (Brown *et al.*, (1997)). Internal standards (d7, section 2.11.1) were added and oxysterols extracted (400 rpm, 2 min, RT) into the solvent mixture (2x). After the phase separation (1000 g, 4°C, 10 min), total lipids were collected in the solvent, evaporated under argon, re-dissolved in toluene:ethyl acetate and processed further as described for plasma samples (section 2.11.1).

### **2.11.3. Extraction From Subcellular Fractions**

Extraction of oxysterols from cytosols or microsomal preparations (200 µg/ml), was achieved as described for plasma (section 2.11.1) with the single exception that 200µl of the sub-cellular fraction was used as the starting material.

### **2.11.4. Quantitation**

To determine concentrations of oxysterols in a mix, a standard curve was produced using a range of stock solutions of 7-oxysterols (0.005 – 200 µM) and cholesterol (0.05 – 2 mM), by adding each stock (10 µl) into H<sub>2</sub>O (500 µl) instead of sample. The internal standards (hepta-deuterated isomers, d7) were added as described (section 2.11.1) and processed identically. The values obtained were adjusted for the initial volume used in order to calculate the measurements in ng/ml plasma or ng/mg protein, as appropriate.

## **2.12. Liquid Chromatographic Analyses Of Steroid And Oxysterols**

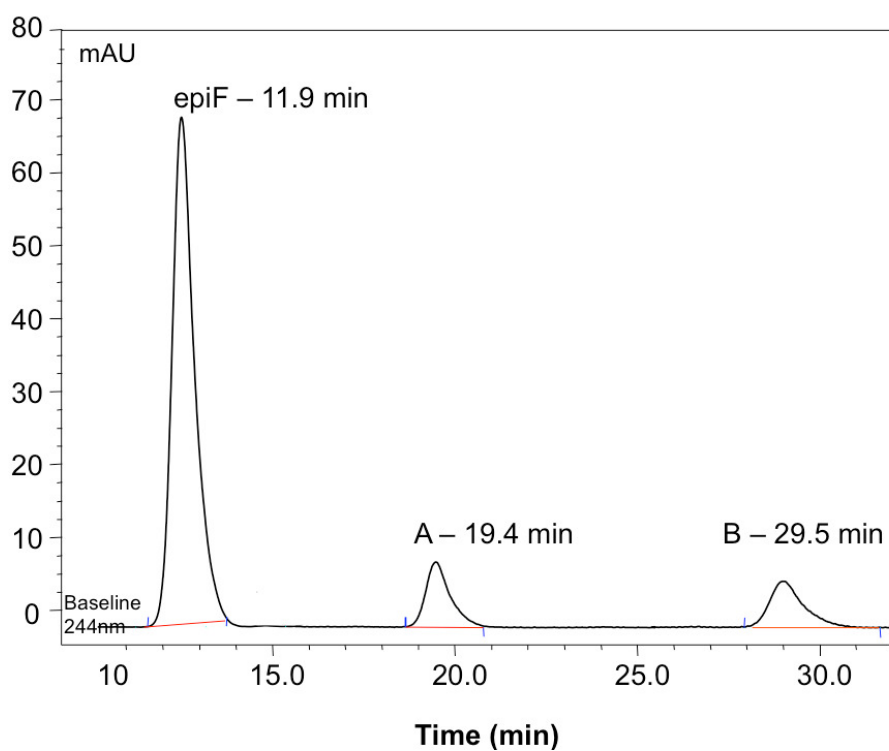
Radiolabelled substrates and products were detected by HPLC with online scintillation counting (HPLC – radiodetection). Unlabelled steroids and oxysterols were detected by HPLC with UV detection (HPLV – UV) or by GC/MS. When dried residues (from the assays described in sections 2.5 – 2.8) were analysed by HPLC–UV, the identity of the peaks was also confirmed by GC/MS. Because components of DMEM caused interference with the HPLC–UV trace of 7βOHC, samples from cell-culture experiments were analysed by GC/MS only. The analytical conditions of chromatographic analyses were established and subsequent detection was performed as described (sections 2.12.1 – 2.13).

### 2.12.1. HPLC With UV-Detection

Unlabelled steroids and oxysterols were analysed using an HPLC-UV (Dionex, UK) system equipped with the photodiode array detector (PDA-100) with a UV/visible detection range ( $\lambda$ 192 - 600 nm). The system comprised an autosampler (ASI-100) and a mobile phase pump (P680), linked to a C18 SunFire column (column length 15 cm, internal diameter 4.6 mm, pore size 5  $\mu$ m, Waters, Edinburgh, UK) and column heater (Igloo-Cil). Spectra for all compounds were recorded in 3D-scan mode (retention time vs. absorbance value vs. wavelength) and their absorption maxima and elution times determined for subsequent analysis. The system was controlled by the Chromeleon computer programme (v6.7, Dionex, UK).

#### 2.12.1.1. *Elution Of Steroids*

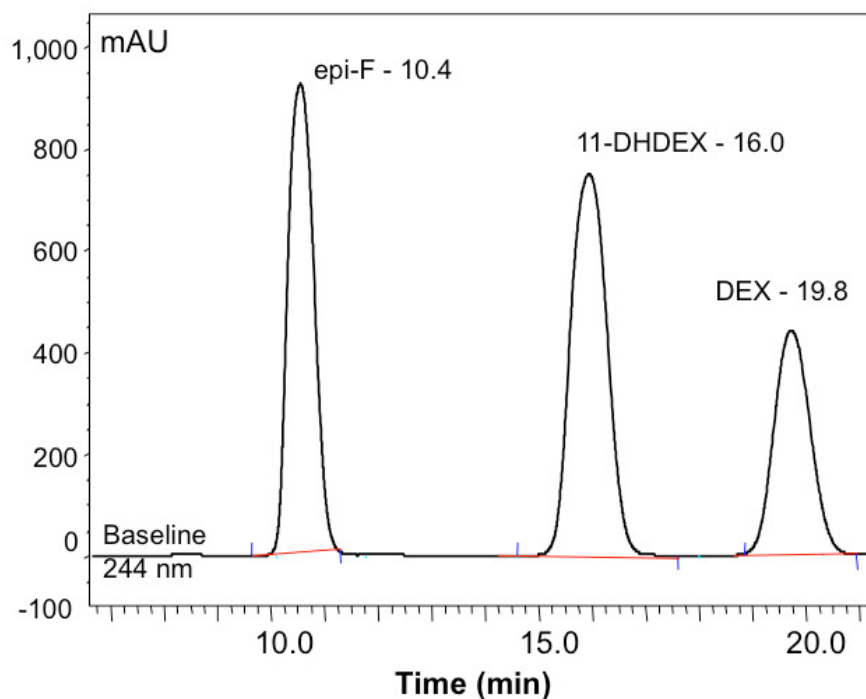
Steroidal extracts containing A and B were dissolved in mobile phase (0.45 ml, 60% water : 15% acetonitrile : 25% methanol) and an aliquot (120 – 200  $\mu$ l) of each was injected into the Dionex HPLC UV-system. The total run time for steroid separation was 35 min at 35°C and 1.0 ml/min flow rate. Elution times of epi-F, A and B were typically 11.9, 19.4 and 29.5 min respectively. Elution times of epi-F, 11-DHDEX and DEX were typically 10.4, 16 and 19.8 min, respectively using 55% water : 20% acetonitrile : 25% methanol, mobile phase at 40°C and with 1.5 ml/min flow as shown in Figures 2.12 – 1 and 2.12 – 2.



**Figure 2.12-1 HPLC chromatogram of unlabelled steroid standards.**

11 $\alpha$ -hydrocortisone (epi-F), 11-Dehydrocorticosterone (A) and corticosterone (B) were detected at 244 nm and separated at 35 °C using water 60% : acetonitrile 15% : methanol 25% mobile phase, at a flow rate of 1 ml/min. mAU, Mili ampere units mV; HPLC, High performance liquid chromatography.



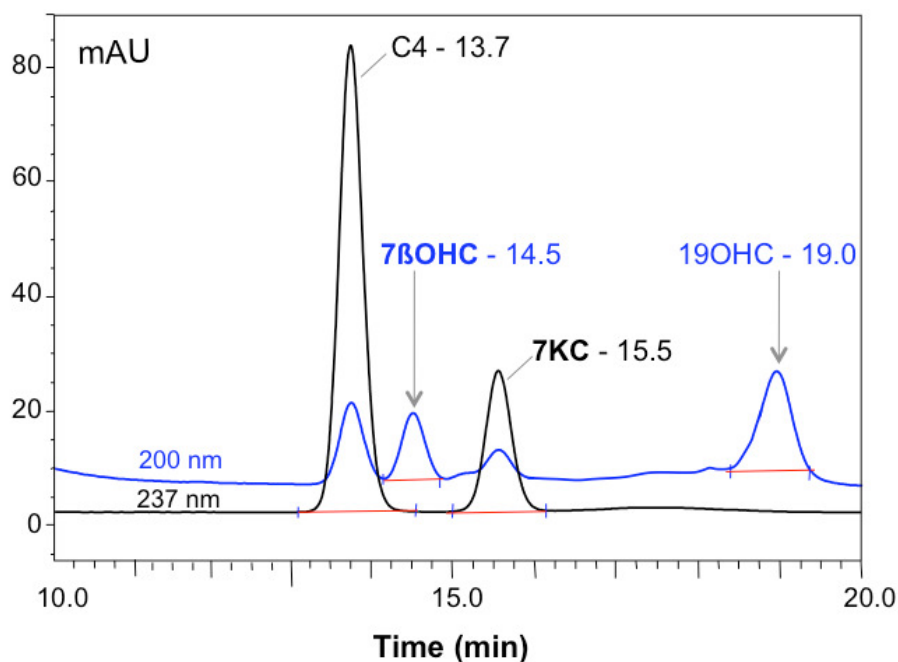


**Figure 2.12-2 HPLC chromatogram of unlabelled steroid standards.**

Dexamethasone (DEX), 11-dehydrodexamethasone (11-DHDEX) and 11 $\alpha$ -hydrocortisone (epi-F) were detected at 244 nm and separated using water 55% : acetonitrile 20% : methanol 25% mobile phase (40°C, flow rate of 1.5 ml/min). mAU, Milli ampere units, mV; HPLC, High performance liquid chromatography.

#### 2.12.1.2. *Elution Of Oxysterols*

Evaporated residues of oxysterol extracts were dissolved in mobile phase (0.45 ml, 5% water : 95% acetonitrile) and an aliquot (150 – 200  $\mu$ l) was injected onto the Dionex HPLC UV-system. Oxysterols were eluted under isocratic conditions at a flow rate of 1.0 ml/min at 24°C. The total run time separation of oxysterols was 20 min. Typical individual elution times were:  $R_t$  14.5 min (7 $\alpha$ OHC),  $R_t$  14.9 min (7 $\beta$ OHC) and  $R_t$  16.4 min (7-KC), and for their internal standards  $R_t$  12 min (C4), and 19 min, (19-OHC) as seen in Figure 2.12 – 3. Where necessary for the separation of 7 $\alpha$ OHC and 7 $\beta$ OHC in the same assay, the peaks were also resolved using GC/MS (section 2.13) and absolute peak identity confirmed as shown (section 2.13).



**Figure 2.12-3 HPLC chromatogram of unlabelled oxysterol standards.**

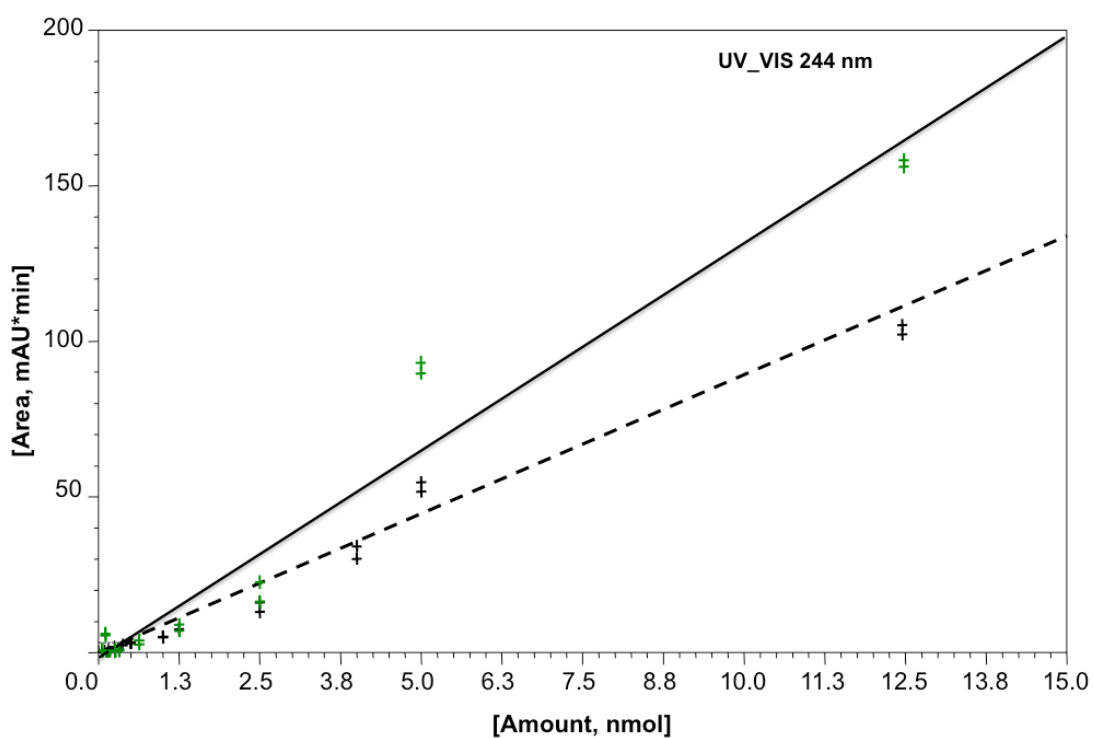
All oxysterols were detected between 200-240 nm. 7-Ketocholesterol (7-KC) and 4-cholestenone (C4) exhibited absorption maxima at 237 nm (black line) and 7β-hydroxycholesterol (7βOHC) and 19-hydroxycholesterol (19-OHC) at 200 nm (blue line). All oxysterols were separated at 24°C using water 5% : acetonitrile 95% mobile phase at a flow rate of 1 ml/min. mAU, Milli ampere units, mV; HPLC, High performance liquid chromatography.

### 2.12.1.3. *Detection Of Steroids And Oxysterols*

Peaks were less than 1.5 min wide and peak height was 50x background. All steroids (epi-F, A, B, DEX and 11-DEX) were detected at 244 nm. Oxysterols were detected at 237 nm (7-KC and C4) and 200 nm (hydroxy-cholesterols)

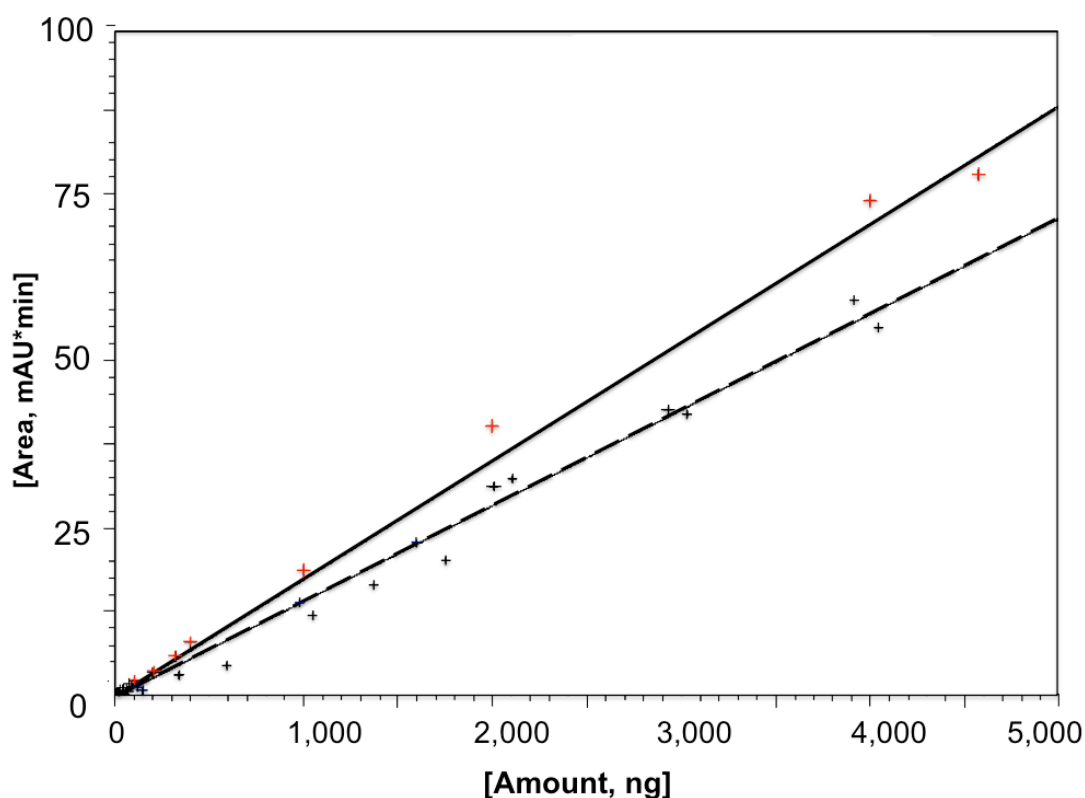
#### 2.12.1.4. **Quantitation**

A standard curve was produced with each experiment to determine concentrations of oxysterols. Standard curves were prepared using a range of stock solutions (0.2 – 200  $\mu\text{M}$ ) by adding a stock (10  $\mu\text{l}$ ) to  $\text{H}_2\text{O}$  (500  $\mu\text{l}$ ) to obtain minimum of eight points. The quantities of oxysterols obtained were adjusted for the initial volume used in order to calculate the measurements in  $\text{nmol/ml}$  assay. Standard curve samples containing known concentrations of analyte were exposed to the same procedures as the samples under investigation. The dried residues were dissolved in the relevant mobile phase (60% water : 15% acetonitrile : 25% methanol for steroids; and 95% acetonitrile : 5% water for oxysterols). An internal standard, which absorbed at the same absorbance maximum as other steroids (244 nm), was used (e.g. epi-F to quantify A and B). If analytes within same assay absorbed light at two different wavelengths, then two internal standards were used (e.g. C4 at 237 nm with 7-KC and 19OHC at 200 nm with 7 $\alpha$ OHC and 7 $\beta$ OHC). After confirming the identity of peaks of interest and related internal standards, areas under the peaks were integrated. For each amount on the standard curve, the mean of duplicates was calculated for the ratios of peak areas over that of the related internal standard and plotted against the known amount. Regression lines were fitted and deemed acceptable if  $R \geq 0.99$ . Steroid or sterol peaks of unknowns were integrated in the same way and ratios of area under the peak to internal standard were interpolated onto the standard curve to obtain the amount of analyte present. In the case of enzyme kinetics, the amount of product (pmol) was divided by time of incubation (min) and specific protein concentration (mg) used in the assay. This value was used as a measure of the reaction velocity (pmol/mg/min). Exemplar standard curves for quantitation of steroid and oxysterols are shown (Figures 2.12 – 4 and – 5).



**Figure 2.12-4 Standard curve for quantitation of steroids detected by HPLC–UV.**

The standard curves representing the ratio of area of peaks 11-dehydrocorticosterone/epi-F (dashed line) and corticosterone/epiF (full line) plotted against known amounts of 11-dehydrocorticosterone and corticosterone standards (nmol) at 244nm. The calibration lines were generated by linear regression. epiF, 11 $\alpha$ -hydrocortisone.



**Figure 2.12-5 Standard curve for quantitation of 7-oxysterols detected by HPLC–UV.**

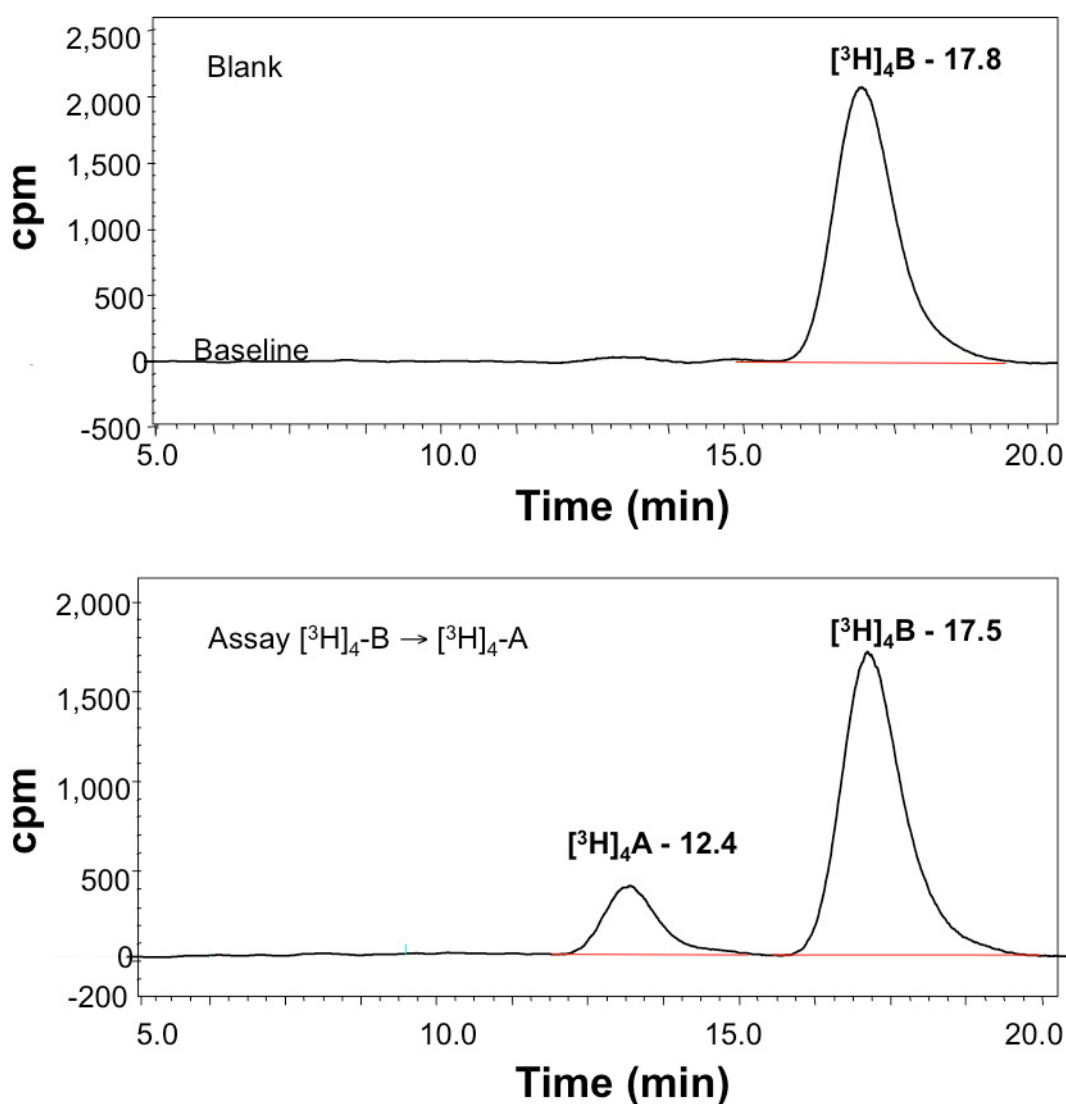
The Standard curves representing the area of peak ratios 7-ketocholesterol/C4 (full line, 237 nm) or 7 $\beta$ -hydroxycholesterol/19OHC (dashed line, 200 nm) plotted against known amounts of 7-ketocholesterol or 7 $\beta$ -hydroxycholesterol (ng) standards. The calibration lines were generated by linear regression. C4, 4-cholesten-7 $\alpha$ -ol-3-one; 19-OHC, 19-hydroxycholesterol.

### **2.12.2. HPLC With Radio-Labelled Detection**

Radio-labelled steroids and sterols were separated using reverse phase HPLC (Waters, UK). The system comprised an autosampler (717 plus) and a mobile phase pump (pump600, Berthold, UK), equipped with C18 Sunfire Symmetry column (column length 15 cm, internal diameter 4.6 mm, pore size 5  $\mu$ m, Waters, Edinburgh, UK), column heater, and radioflow detector (LB509, Berthold) linked to a scintillation fluid pump. The system was controlled by the Chromeleon computer software (v6.7, Dionex, UK).

#### **2.12.2.1. Elution Of Steroids**

Dried residues of steroidal extracts were dissolved in 0.8 ml mobile phase (60% water : 15% acetonitrile : 25% methanol) and an aliquot (60 - 100  $\mu$ l) of each was injected into the HPLC system. The total run time for steroid separation was 35 min at 35°C and 1.0 ml/min flow rate. Elution times of [ $^3$ H] $_4$ -A and [ $^3$ H] $_4$ -B were typically 12.4 and 17.5 min respectively (Figure 2.12 – 6). An additional peak at 13 min (always less than 6% radioactivity) was present in some assays, when [ $^3$ H] $_4$ -A was incubated with microsomal protein, but not in the ‘Blank’ control samples (lacking protein). This peak was believed to be a metabolite of [ $^3$ H] $_4$ -A (e.g. 20 $\beta$ -dehydro-11-dehydrocorticosterone), but a standard was not available for confirmation.



**Figure 2.12-6 HPLC radio-chromatogram of  $[^3\text{H}]_4$ -steroid detection.**

Steroids ( $[^3\text{H}]_4$ -11-dehydrocorticosterone, A and  $[^3\text{H}]_4$ -corticosterone, B) were extracted and dissolved in mobile phase (0.8 ml) of 60% water : 15% acetonitrile : 25% methanol (v/v/v). Sample was then injected into an HPLC system and steroids eluted using the same mobile phase at 1.0 ml/min flow rate, at 35°C. Separation of  $[^3\text{H}]_4\text{-A}$  and  $[^3\text{H}]_4\text{-B}$  was achieved (bottom) with the approximate retention times (Rt 12.4 min and 17.5 min respectively). Control sample ('Blank') had no protein and, as expected, only substrate  $[^3\text{H}]_4\text{-B}$  was detected (top). HPLC, High performance liquid chromatography.

#### **2.12.2.2. Elution Of Oxysterol**

Evaporated residues of radio-labelled oxysterol extracts were dissolved in mobile phase (0.8 ml, 15% water : 85% acetonitrile) and an aliquot (200 µl) of each was injected onto the HPLC. Oxysterols were eluted under isocratic conditions at a flow rate of 1.5 ml/min at 28°C. The total run time for oxysterol separation was 25 min with individual elution times of [ $^3\text{H}$ ] $_3$ -7-KC and [ $^3\text{H}$ ] $_3$ -7 $\beta$ OHC typically at 16 and 19 min, respectively (Figure 2.12– 7).

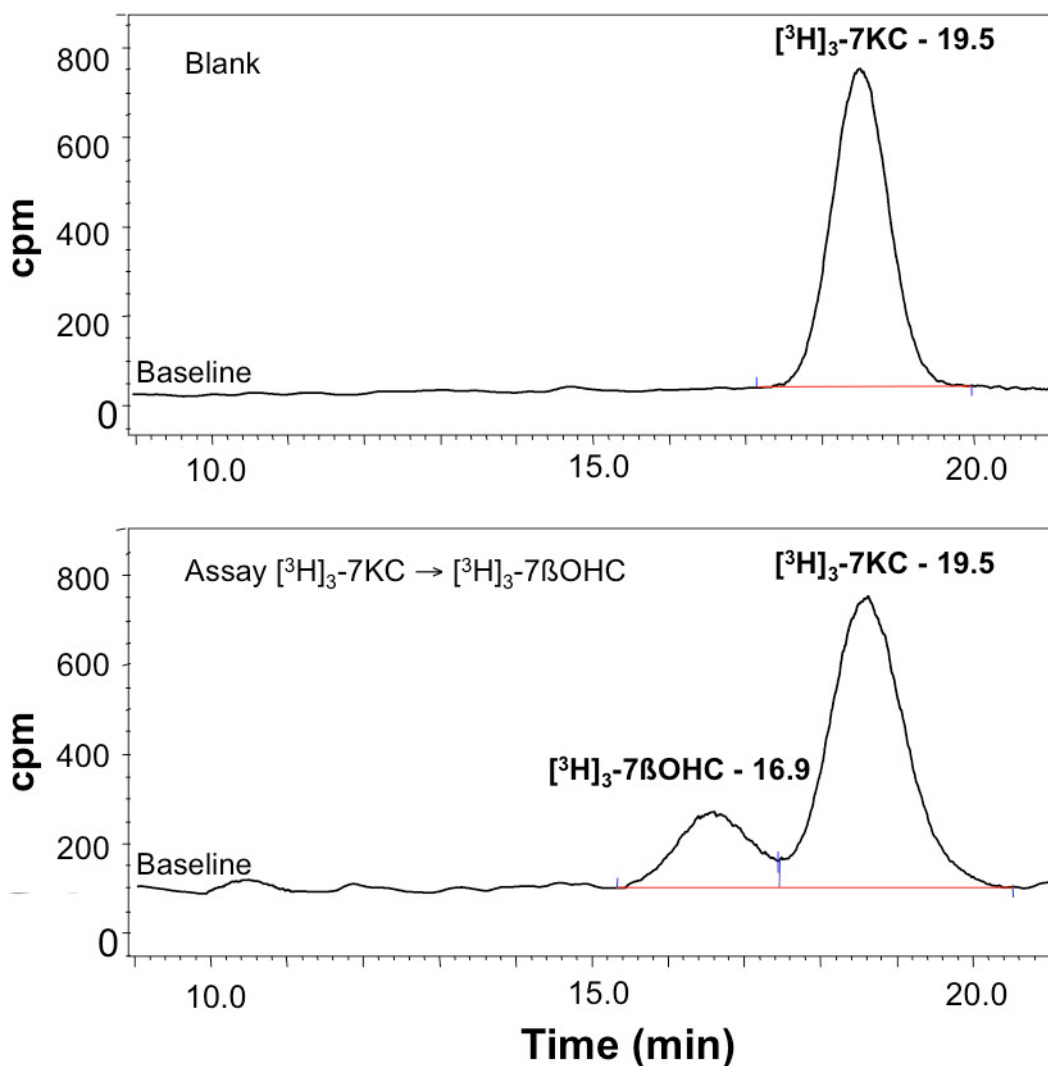
#### **2.12.2.3. Detection Of Steroids And Oxysterols**

Detected peaks were less than 2 min wide and considered detectable if peak height was 5x background.

#### **2.12.2.4. Quantitation**

Steroids and sterols were quantified by on-line liquid scintillation counting (2 ml/min scintillation fluid). Detected peaks were integrated and relative peak area (%) recorded for the substrate and product. The amount of product formed (pmol) from a known amount of substrate (pmol) was then calculated and the values expressed per time of incubation (min) and specific protein concentration (mg) used in the assay. This value was then used as a measure of reaction velocity (pmol/mg/min).





**Figure 2.12-7 HPLC radio chromatogram of  $[^3\text{H}]_3$ -oxysterol detection.**

Oxysterols ( $[^3\text{H}]_3$ -7-Ketocholesterol, 7-KC and  $[^3\text{H}]_3$ -7 $\beta$ -hydroxycholesterol, 7 $\beta$ OHC) were extracted and dissolved in 0.8 ml mobile phase of 15% water : 85% acetonitrile (v/v). An aliquot (200  $\mu\text{l}$ ) was then injected into an HPLC system and oxysterols eluted using the same mobile phase at 1.5 ml/min flow rate, at 28  $^{\circ}\text{C}$ .  $[^3\text{H}]_3$ -7 $\beta$ OHC and  $[^3\text{H}]_3$ -7-KC were eluted at 16.9 and 19.5 min, respectively (bottom). The control sample ('Blank') had no protein and, as expected, only substrate  $[^3\text{H}]_3$ -7-KC was detected (top). HPLC, High performance liquid chromatography.

## 2.13. Gas Chromatography-Mass Spectrometry (GC/MS)

Gas chromatography-mass spectrometry (GC/MS) has been (Dzeletovic *et al.*, (1995b), Dzeletovic *et al.*, (1995a)) and is (Endo *et al.*, (2008); Larsson *et al.*, (2007)) used extensively for plasma and tissue oxysterol analysis. The advantage of this technique is an exquisite power of separation. In addition the electron impact (EI) spectra provide further information regarding identity of analytes. The ultimate sensitivity in detection of 7-oxysterols is achieved when analysis is performed with the MS instrument operating in selective ion monitoring mode (SIM) combined with the capillary GC column for separation. However, pre-treatment (derivatisation) of the samples is necessary to render the oxysterols volatile.

### 2.13.1. Gas Chromatography Conditions

A capillary gas chromatograph (Trace GC, Thermo) coupled to an ion-trap, Polaris Q (Thermo, Hemel Hempstead, UK) mass spectrometer (MS) and equipped with a BPX5 capillary column (25 m, 0.32 mm internal diameter and 0.25  $\mu$ m film thickness, from SGE, Alva, UK) was used for analysis. Helium was used as the carrier gas at a flow rate of 1 ml/min. The Polaris Q MS was operated using Xcalibur (v10.0) software.

The GC conditions were optimized as follows. The initial temperature was optimized at 180°C, near the boiling point of decane (174°C) in which samples were finally dissolved. The injector temperature was optimized at 270°C. Mass spectra were recorded at an electron ionisation energy of 70eV with an ion source temperature of 200°C and an interface temperature of 250°C. All samples were prepared for injection in decane containing 2% BSTFA (80  $\mu$ l) to ensure stability of derivatized sterols.

### 2.13.2. Optimized Method

The following final two methods were used in this thesis for analysis and oxysterol detection in the samples from tissue culture experiments (Method 1) and in biological fluids and tissues (Method 2).

#### *Method 1 – Analysis of cell culture samples.*

BSTFA and 1 % pyridine, 100  $\mu$ l (Endo *et al.*, (2008)) were added to the dried residue (60°C, 30 min) under argon in silanized reacti vials (as in section 2.2.2). Following derivatisation, samples were dissolved in decane (2% BSTFA) and transferred to GC/MS amber vials with inserts. The derivitised oxysterols were eluted using the following oven programme: initial temperature 180°C (1min), increased by 20°C/min until 300°C (1min) and then by 5°C/min until 330°C (8 min). The oven was then cooled by -10°C/min to 250°C (1 min).

#### *Method 2 – Analysis of biological fluids and tissues*

The dried residue (60°C) was incubated (30 min) with 350  $\mu$ l pyridine : HMDS : TMCS (3:2:1, v/v/v; Dzeletovic *et al.*, (1995b), Larsson *et al.*, (2007)) under argon and derivatized samples dissolved in 2% BSTFA in decane before being transferred into GC/MS amber vials with inserts. The derivitised oxysterols were eluted using a method modified from Larsson *et al.*, 2007, oven programme – initial temp: 180°C (1min), increased by 35°C/min until 270°C (1min), then by 4°C/min until 300°C and temperature maintained for 12 min.

### 2.13.3. Detection Of Oxysterols And Cholesterols

Both GC chromatograms and mass spectral fragmentation patterns of trimethylsilyl-ether derivatives of analytes were obtained using authentic standards. The mixture of d7 standards (1ml) was prepared from d7-7 $\beta$ OHC (200 ng/ $\mu$ l, 50  $\mu$ l) and d7-7-KC (200 ng/ $\mu$ l, 200  $\mu$ l) in ethanol containing 50  $\mu$ g/ml BHT. Of this solution, an aliquot (50  $\mu$ l) was used for quantifying oxysterols (as in section 2.13.4).

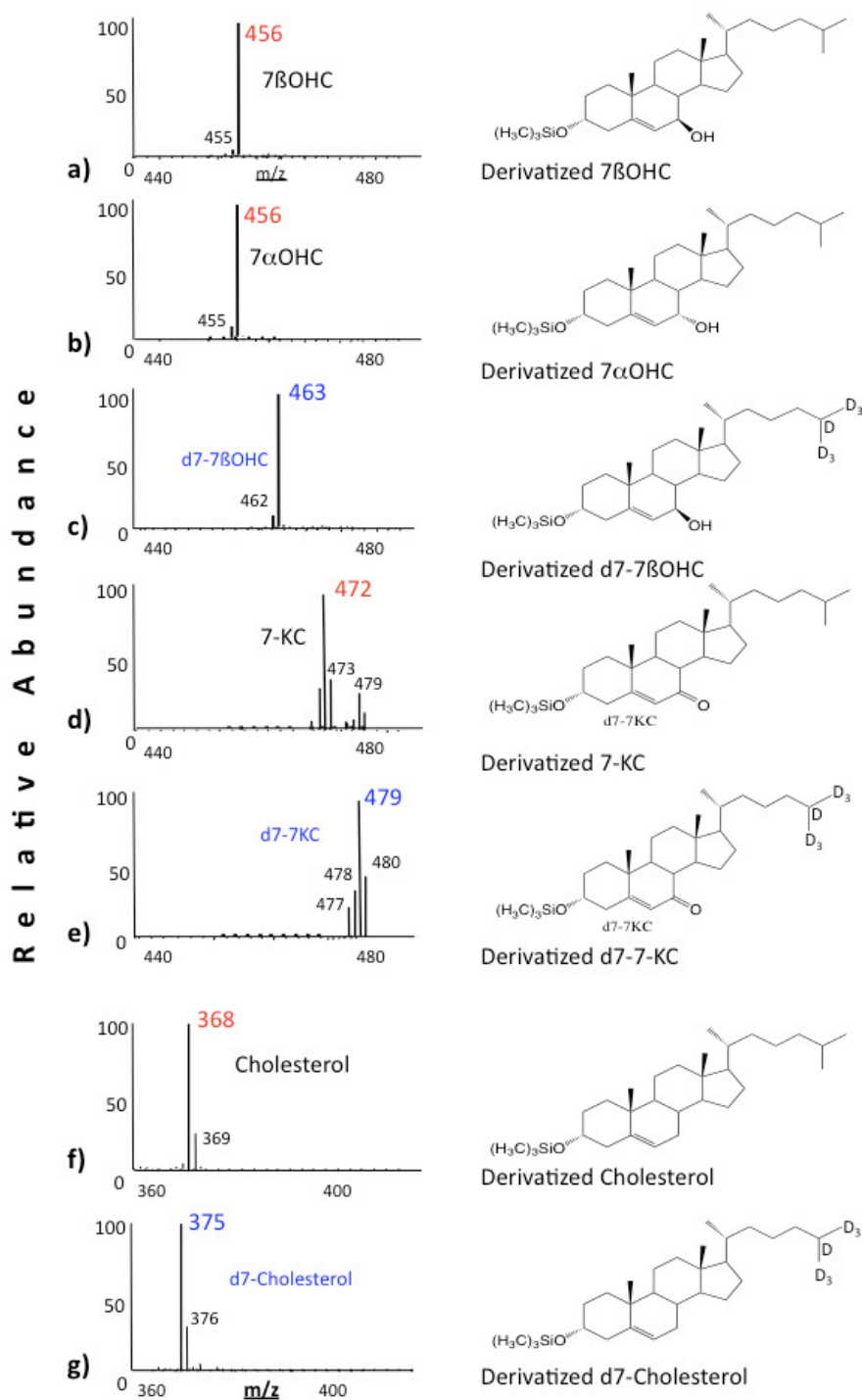
Full and SIM scans of ions were generated for all derivatized compounds: examples of spectra and SIM mass chromatograms of the derivatized-7oxysterols and cholesterol are shown in Figures 2.13 – 1 and –2. Derivatized 7 $\alpha$ OHC and 7 $\beta$ OHC had base peaks of  $m/z$  456. Derivatized-7-KC had a base peak at  $m/z$  472, while cholesterol yielded  $m/z$  368. All of the compounds had characteristic retention times (Table 2 - 1).

#### **2.13.4. Quantitation**

Limits of detection were assigned as 3:1 signal to noise ratio. The area of the peak of the analyte was integrated and divided by that of the internal standard and compared to ratios generated from the standard curve. Lines of best fit were drawn to generate a standard curve of peak area ratio versus known amount (acceptable if  $R \geq 0.99$ ) and quantities of the unknown interpolated from the line. This value was used as a measure of the reaction velocity (pmol/mg/min) and levels (ng/ml fluid or ng/mg tissue). The amount of product detected in samples (pmol) was expressed over the time of incubation (min) and specific protein concentration (mg) in the assay or related to the volume of fluid/tissue analysed.

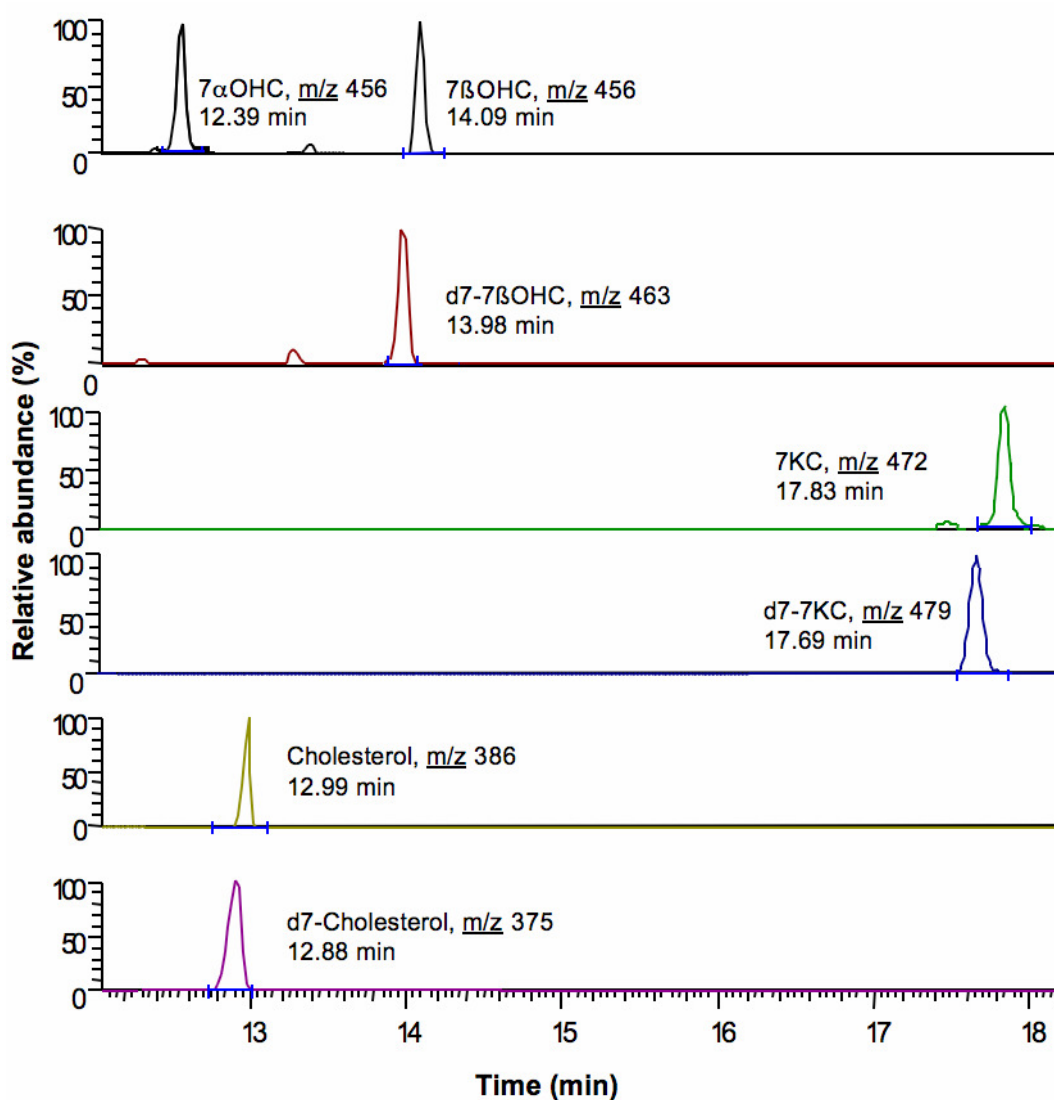
#### **2.14. Statistical Analysis**

All data in this thesis are presented as mean  $\pm$  standard error of the mean (SEM) with statistical analysis stated in individual chapters. Student's t-test, one-way or two-way ANOVA with Dunnett's or Tukey's *post hoc* test were performed, as appropriate, and data were analyzed using GraphPad Prism software v5.0 (GraphPad Software Inc. San Diego, USA, [www.graphpad.com](http://www.graphpad.com)). Statistical significance was assumed when  $p < 0.05$ .



**Figure 2.13-1 GC/MS spectra of derivatized 7-oxysterols and cholesterol.**

Following analysis by gas chromatography mass spectrometry (GC/MS), the mass to charge ( $m/z$ ) ratios of ions generated by the individual compounds were plotted against their relative abundance (%). Analysis was operated in full scan mode to determine the base peaks for TMS-7-oxysterols: **a)** 7 $\beta$ OHC,  $m/z$  456; **b)** 7 $\alpha$ OHC,  $m/z$  456; **c)** d7-7 $\beta$ OHC,  $m/z$  463; **d)** 7-KC,  $m/z$  472; **e)** d7-7-KC,  $m/z$  479), and TMS-cholesterols: **f)** Cholesterol,  $m/z$  368; **g)** d7-cholesterol,  $m/z$  375.



**Figure 2.13-2 GC/MS chromatograms of derivatised 7-oxysterols and cholesterol.**

The gas chromatography (GC) mass spectrometer was programmed in selective ion monitoring (SIM) mode to detect the following ions of TMS-7-oxysterols: 7 $\alpha$ -hydroxycholesterol, 7 $\alpha$ OHC ( $m/z$  456, 12.39 min); 7 $\beta$ -hydroxycholesterol, 7 $\beta$ OHC ( $m/z$  456, 14.09 min); deuterium-labeled 7 $\beta$ OHC, d7-7 $\beta$ OHC ( $m/z$  463, 13.98 min); 7-ketcholesterol, 7-KC ( $m/z$  472, 17.83 min); deuterium-labeled 7-KC, d7-7-KC ( $m/z$  479, 17.69 min); Cholesterol ( $m/z$  386, 12.99 min) and deuterium-labeled cholesterol, d7-cholesterol ( $m/z$  375, 12.88 min). The relative abundance of each derivatised-compound was determined at an elution time (min) as given.  $m/z$ , mass to charge ratio.

**Table 2-1 GC/MS detection of derivatized oxysterol and cholesterol ions.**

<b>Ion</b>	<b>m/z</b>	<b>Isolation width (amu)</b>	<b>Approximate retention time (min)</b>
<b>19OHC</b>	353	2.0	11.9
<b>7<math>\alpha</math>OHC</b>	456	4.0	12.83
<b>d7 – Cholesterol</b>	375	6.0	12.88
<b>Cholesterol</b>	368	6.0	12.99
<b>d7 – 7<math>\beta</math>OHC</b>	463	4.0	13.98
<b>7<math>\beta</math>OHC</b>	456	4.0	14.09
<b>d7 – 7-KC</b>	479	6.0	17.69
<b>7-KC</b>	472	6.0	17.83
<b>C4</b>	472	6.0	20.1

The most abundant ions of trimethylsilyl derivatives of cholesterol and oxysterols (m/z) were detected in the selective ion monitoring mode (SIM) mode by gas chromatography mass spectrometry (GC/MS) analysis. m/z, mass to charge ratio; Amu, Atomic mass units.

## **Chapter 3. Kinetics Of 7-Oxysterol Metabolism By 11 $\beta$ HSD1**



### 3.1. Introduction

A novel role for 11 $\beta$ HSD1 in metabolising 7-oxysterols, was first demonstrated in 2004 (Hult *et al.*, 2004; Schweizer *et al.*, 2004). Prior to this, Song *et al.*, (1998) showed that an NADP<sup>+</sup> dependent 7 $\alpha$ -hydroxycholesterol dehydrogenase (7 $\alpha$ HCD) existed in hamster liver microsomes, which generated 7-ketocholesterol (7-KC) from either 7 $\alpha$ -hydroxycholesterol (7 $\alpha$ OHC) or 7 $\beta$ -hydroxycholesterol (7 $\beta$ OHC). Sequence analysis of 7 $\alpha$ HCD at this time indicated the protein under investigation exhibited a high degree of homology to human 11 $\beta$ HSD1 (Song *et al.*, 1998). Subsequent work by Hult *et al.* and Schweizer *et al.*, in 2004 demonstrated direct *in vitro* and *in vivo* evidence that human and rat 11 $\beta$ HSD1 catalyse the inter-conversion of 7-KC and 7 $\beta$ OHC. Cross-species comparison of reactions catalysed by 11 $\beta$ HSD1, showed that 7-KC was reduced to 7 $\beta$ OHC only, except in hamster in which 7 $\alpha$ OHC was also formed (Arampatzis *et al.*, 2005). At the onset of the work described in this thesis, emerging literature suggested a metabolism of 7-oxysterols by murine 11 $\beta$ HSD1 (Hult *et al.*, 2004) but confirmation that 11 $\beta$ HSD1 was the only enzyme catalysing this reaction *in vivo* had yet not been obtained.

*In vivo*, the predominant direction of 7-oxysterol metabolism by 11 $\beta$ HSD1 was anticipated to be reduction, as with glucocorticoids, converting 7-KC to 7 $\beta$ OHC. Indeed, reduction of 7-KC was assigned to the action of rat hepatic 11 $\beta$ HSD1 (Schweizer *et al.*, 2004). However Maeda *et al.*, (2006) reported that microsomal 11 $\beta$ HSD1 utilises 7 $\beta$ OHC to form 7-KC in all of the vertebrates tested, but the opposite reaction occurs only in rats; again the set-point for the equilibrium had not been assessed in mouse. The conversion of glucocorticoids by 11 $\beta$ HSD1 *in vivo* is dependent on availability of endogenous cofactor, NADPH, generated by hexose-6-phosphate dehydrogenase (H6PDH). Both enzymes are localized to the lumen of the ER and their direct physical interaction is required for 11 $\beta$ HSD1 to act as a reductase (Atanasov *et al.*, 2008; Atanasov *et al.*, 2004). Importantly, in mice lacking H6PDH, glucocorticoid regeneration by 11 $\beta$ HSD1 was abolished (Lavery *et al.*, 2006) but a similar effect on metabolism of 7-oxysterols has not been investigated.

Indeed there are not currently any reports considering the role of H6PDH in cofactor supply for metabolism of 7-oxysterols by 11 $\beta$ HSD1. Studies with the mutant 11 $\beta$ HSD1 protein have only demonstrated *in vitro* a crucial role of the N-terminal region of the enzyme in determining its luminal orientation in ER. Such orientation of 11 $\beta$ HSD1 within the ER is essential for efficient oxidation of cortisol as well as reduction of 7-KC (Frick *et al.*, 2004).

In addition to proving the metabolic benefit of 11 $\beta$ HSD1 inhibition, transgenic mice lacking 11 $\beta$ HSD1 (11 $\beta$ HSD1<sup>-/-</sup>) have provided a valuable resource to determine the precise role of murine 11 $\beta$ HSD1 in the metabolism of glucocorticoids (Kotelevtsev *et al.*, 1997). Now, the same transgenic model provides a unique source for examining the role of murine 11 $\beta$ HSD1 in the metabolism of atherogenic 7-oxysterols.

### 3.2. Research Hypothesis And Aims

Key questions for the work in this chapter were: (1) can murine 11 $\beta$ HSD1 catalyse the inter-conversion of 7-oxysterols and if so, (2) what is the predominant direction of the reaction. Complementary *in vitro* assays were used to clarify kinetics of this inter-conversion. Specific aims were:

- To model, *in silico*, the potential interactions of 7-oxysterols with murine and human 11 $\beta$ HSD1 proteins;
- To determine whether murine 11 $\beta$ HSD1 (m11 $\beta$ HSD1) has the ability to catalyse the inter-conversion of 7-oxysterols, and determine the nature of the preferred substrate;
- To compare the kinetic parameters of metabolism of 7-oxysterols by m11 $\beta$ HSD1 with those of glucocorticoids.

### 3.3. Methods

#### 3.3.1. 3-D Modelling Of 7-Oxysterols Bound To 11 $\beta$ HSD1

The following structures were used as templates to investigate the interactions of 7-KC, 7 $\alpha$ OHC and 7 $\beta$ OHC with 11 $\beta$ HSD1. All protein structures used were freely available from PubMed ([www.pubmed.org](http://www.pubmed.org), NCBI) and the protein database (PDB, <http://www.rcsb.org/pdb/Welcome.do>, NCBI). Sequence alignment was performed using ClustalW2 program (Larkin *et al.*, 2007) available from [www.ebi.ac.uk/tools/clustalw2/index.htm](http://www.ebi.ac.uk/tools/clustalw2/index.htm) (accessed March, 2010). Accession numbers of the 11 $\beta$ HSD1 sequences are as follows: human NM\_005525, mouse NM\_008288, hamster, AY519498.

The 3-D structures of crystalised murine 11 $\beta$ HSD1 (m11 $\beta$ HSD1, PDB ID: 1Y5M), or m11 $\beta$ HSD1 in complex with corticosterone (PDB ID: 1Y5R, (Zhang *et al.*, 2005)), as well as human 11 $\beta$ HSD1 (h11 $\beta$ HSD1, PDB ID: 2BEL) in complex with NADP and carbenoxolone (Wu *et al.*, 2006), were used for *in silico* modelling. Comparisons were made between the orientations of human and murine structures. The structure of m11 $\beta$ HSD1 (1Y5R) contains both corticosterone and NADP<sup>+</sup> (Zhang *et al.*, 2005), and was used further to superimpose C7-oxysterol analogues.

In addition, the 1FMC structure of 7 $\alpha$ -hydroxysteroid dehydrogenase (EC 1.1.1.159, 7 $\alpha$ HSD) in complex with 7-oxo glycochenodeoxycholic acid was used (Tanaka *et al.*, 1996), as it offered an excellent template for modelling the 7 $\alpha$ -hydroxyl group, thus allowing alignment and positioning of 7 $\alpha$ - and 7 $\beta$ -hydroxyls and 7-keto groups. 7 $\alpha$ HCD was superimposed with 11 $\beta$ HSD1 and the binding of 7-oxo-glycochenodeoxycholic acid was merged into the structure of 11 $\beta$ HSD1 as described later (section 3.4.2).

The energy map of substrate binding into the active site of 11 $\beta$ HSD1 protein were obtained using Autodock (freely available from <http://autodock.scripps.edu>, GNU General Public License). This program uses 3-D protein structure, as imported from

PDB, to calculate energy maps for binding of a small molecule. Positioning the ligand into the site of interest is carried out by an algorithm, which aimed to find minimal energy conformations of the ligand in the pocket by rotating and translating the molecules. The results of this type of docking are then clustered into groups. The “middle” conformation for each cluster is then written out as a result for that cluster along with a predicted energy of interaction. Further LIDAEUS software (Taylor *et al.*, 2008) kindly provided by Dr Steve Shave (University of Edinburgh, Laboratory of enzyme crystallography) was used to define the binding pocket. For visualising the structures of enzyme and compounds, the molecular graphics software, PyMOL (open source, DeLano Scientific LLC) was used, kindly provided by Dr Iain McNae (University of Edinburgh, Laboratory of enzyme crystallography). The distances between key functional moieties and catalytic residues in the active site were assessed using the same software.

The 2-D representation of protein-ligand complexes from standard Protein Data Bank file input were performed using LigPlot (Wallace *et al.*, 1995). To distinguish between the steric orientation of 7 $\alpha$ - and 7 $\beta$ -hydroxycholesterols, the 2-D diagrams for each molecule were created with MarvinView by ChemAxon (<http://www.chemaxon.com/products/marvin/marvinview/>, accessed April, 2010) and used with LigPlot. Both, programs and resultant images were kindly provided by Dr Steve Shave (University of Edinburgh, Laboratory of enzyme crystallography).

### 3.3.2. Oxidation Of 7 $\beta$ -Hydroxycholesterol And 7 $\alpha$ -Hydroxy-Cholesterol To 7-Ketocholesterol By m11 $\beta$ HSD1

All oxysterols were prepared in ethanol containing butylated hydroxytoluene (50  $\mu$ g/ml), as described (section 2.2.2). The conversion of 7 $\beta$ OHC and/or 7 $\alpha$ OHC to 7-KC was studied using the following assays and reaction velocities compared with those of conversion of corticosterone (B) to 11-dehydrocorticosterone (A):

*a) Recombinant m11 $\beta$ HSD1 protein* (28  $\mu$ g/ml) was incubated with 7 $\beta$ OHC or 7 $\alpha$ OHC (20  $\mu$ M) or B (20 - 200 nM) and the cofactor NADP<sup>+</sup> (2 mM) as described (sections 2.5.3 and 2.5.2, respectively). The kinetic parameters of metabolism of

each substrate for m11 $\beta$ HSD1 were also determined in the presence of NAD<sup>+</sup> (2 mM) instead of NADP<sup>+</sup>.

**b) HEK cells stably transfected to produce m11 $\beta$ HSD1 protein** were incubated with 7 $\beta$ OHC or 7 $\alpha$ OHC (1  $\mu$ M), as described (section 2.6.3), or with B (30 nM) as in section 2.6.2.

**c) Murine hepatic microsomes (WT)** were used as a source of m11 $\beta$ HSD1 as described (section 2.7.4). Microsomal protein (240  $\mu$ g/ml) was incubated with 7 $\beta$ OHC or 7 $\alpha$ OHC (20  $\mu$ M) or with B (20 – 200 nM) and with 2mM cofactor (NAD<sup>+</sup> or NADP<sup>+</sup>, as required). Similar experiments were performed using microsomes from 11 $\beta$ HSD1<sup>-/-</sup> mice.

**d) Aortic rings (WT)** express both 11 $\beta$ HSD type 1 and type 2 isozymes (Hadoke *et al.*, 2001). In this assay, aortic rings were used to investigate oxidation of 7 $\beta$ OHC or 7 $\alpha$ OHC (1  $\mu$ M) by 11 $\beta$ HSD1, as well as oxidation of B (30 nM), as described (sections 2.8.2 and 2.8.1, respectively). In both assays, samples were incubated with the appropriate substrate and without exogenous cofactor, for 20 h. Similar experiments were repeated using aortic rings from 11 $\beta$ HSD1<sup>-/-</sup> mice which only express 11 $\beta$ HSD type 2 (Christy *et al.*, 2003).

### 3.3.3. Reduction Of 7-Ketocholesterol To 7 $\beta$ -Hydroxycholesterol By m11 $\beta$ HSD1

The conversion of 7-KC to 7 $\beta$ OHC and/or 7 $\alpha$ OHC was studied using the following assays and reaction velocities compared with those of A  $\rightarrow$  B:

**a) Recombinant m11 $\beta$ HSD1** protein (28  $\mu$ g/ml) was incubated with 7-KC (20  $\mu$ M) or A (20 - 200 nM) and the NADPH (2 mM) generating system (sections 2.5.4.1 and 2.5.5). To further explore the specificity of cofactor for this reaction, reduction was performed in the presence of NADH (2 mM) instead of the NADPH generating system.

**b) HEK cells stably transfected to produce m11 $\beta$ HSD1 protein** were incubated (37°C, 16h) with 7-KC (1  $\mu$ M), or A (30 nM) as described (sections 2.6.5 and 2.6.4).

**c) m11 $\beta$ HSD1 protein, obtained upon lysis of HEK293 cells** as in section 2.6.7.1. This reaction was performed using protein from lysed cells (100  $\mu$ g/ml) and radio-labelled substrate ( $[^3\text{H}]_3$ -7-KC, 5 nM) together with unlabelled 7-KC (200 nM, 1  $\mu$ M, 20  $\mu$ M).

**d) Murine hepatic microsomes (WT)** were used as a source of m11 $\beta$ HSD1 as described (section 2.7.4). Protein (260  $\mu$ g/ml) was incubated with 7-KC (1  $\mu$ M or 20  $\mu$ M) or A (20 - 200 nM), and one of the following cofactors, NADH (2 mM), NADPH (2 mM), or an NADPH-generating system (section 2.5.4.1). Similar experiments were performed using microsomes from 11 $\beta$ HSD1<sup>-/-</sup> mice. Using the same assay conditions, the reaction was also performed with permeabilized microsomes; prior addition of allameticine (500  $\mu$ g/ml, 1-2h) was used to induce cationic permeability of the microsomes to allow substrate transport across the membrane (Pressman, 1976).

**e) Aortic rings (WT)** were used to assess reduction of 7-KC (1  $\mu$ M) by m11 $\beta$ HSD1, as well as reduction of A (30 nM). The assay was performed as described (section 2.8.4). Both assays were incubated for 20 h. Similar experiments were performed using aortic rings from 11 $\beta$ HSD1<sup>-/-</sup> mice.

### **3.3.4. Establishing Assay Conditions For 11 $\beta$ HSD1 Bioassays With Steroids And 7-Oxysterols**

Initial experiments were performed with each source of enzyme in order to establish the conditions under which the reaction proceeded with first order kinetics (normally  $\leq 30\%$  conversion). Studies of the time-course of product formation were performed using m11 $\beta$ HSD1 protein (28  $\mu$ g/ml), microsomes (240 – 260  $\mu$ g/ml) or cell lysates (100  $\mu$ g/ml), and the velocity of product formation quantified after 0, 15, 30, 45, 60, 90 and 180 minutes. From these, a single time-point and protein concentration were

selected for subsequent experiments. For assays in which both dehydrogenation and reduction were evident (cells and aortic rings), a single time point (20 h) was chosen to achieve product formation for the reductase reaction (30% conversion) from both substrates; dehydrogenation proceeded on average ~2.5 times slower, reaching ~12% conversion.

### 3.3.5. Michaelis-Menten Analysis Of 11 $\beta$ HSD1 Enzyme Kinetics

Purified recombinant enzyme (14 – 28  $\mu$ g/ml) or preparations of WT hepatic microsomes (240 - 260  $\mu$ g/ml) were incubated (see sections 2.5 and 2.7, with solutions of oxysterols (500 nM - 500  $\mu$ M) or steroids (20 nM - 2  $\mu$ M) representing a wide range of concentrations. Initial reaction velocities were measured, and conditions were chosen to achieve less than 30% of conversion of substrate to product; typically a reaction time of 50 min with recombinant protein (28  $\mu$ g/ml) and 30 min with hepatic microsomes (260  $\mu$ g/ml).

### 3.3.6. Metabolism of 7-Oxysterols By 11 $\beta$ HSD2

The oxidation of 7 $\beta$ OHC or 7 $\alpha$ OHC and reduction of 7-KC by 11 $\beta$ HSD2 was investigated using the following models and the reaction velocities were compared with those of oxidation of dexamethasone (DEX) to 11-dehydrodexamethasone (11-DHDEX), or reduction of 11-DHDEX to DEX:

*a) CHO cells stably transfected to generate h11 $\beta$ HSD1 protein* were incubated with 7 $\beta$ OHC, 7 $\alpha$ OHC or 7-KC (1  $\mu$ M), as described (section 2.9.1).

*b) Kidney homogenates from WT and 11 $\beta$ HSD1<sup>-/-</sup> mice* were used as a source of 11 $\beta$ HSD2 as described (section 2.9.2). Samples containing protein (400  $\mu$ g/ml) were incubated with 7 $\beta$ OHC, 7 $\alpha$ OHC or 7-KC (20  $\mu$ M) and one of the following cofactors, NAD<sup>+</sup>, NADP<sup>+</sup>(both dehydrogenase), NADH or NADPH (both reductase) (2mM) to test for product formation with each appropriate substrate-cofactor combination.

c) *m11 $\beta$ HSD2* activity in aortic ring preparations from *11 $\beta$ HSD1*<sup>-/-</sup> mice was assessed in an assay of oxidation of 7 $\beta$ OHC (1  $\mu$ M) or reduction of 7-KC (1  $\mu$ M). The reaction was performed as described (sections 2.8.2 and 2.8.4). A control assay using aortic rings from WT mice (which express both 11 $\beta$ HSD1 and 11 $\beta$ HSD2) (Christy, 2003; Christy *et al.*, 2003) was performed in the same experiment.

### 3.3.7. Detection Of Oxysterols

Dried extracts from the bioassays described in this chapter were analysed by HPLC or GC/MS.

#### 3.3.7.1. HPLC Analysis Of Oxysterols

All HPLC analyses of steroids and oxysterols were performed using UV- and on-line scintillation detection as described (sections 2.12.1 and 2.12.2).

#### 3.3.7.2. GC/MS Analysis Of Oxysterols

The final method for detection of oxysterols using gas chromatography with mass spectrometry (GC/MS) is described in section 2.13.2. Selected ions were used to detect each of the specific derivatised compounds as described (section 2.13.3) and these are detailed in Table 2 - 1.

The initial approach to detection of oxysterols involved determination of running conditions, retention time, peak abundance, peak characteristics and separation from other oxysterols. A full scan of ions generated by each compound was recorded initially in the mass range of  $m/z$  200-700 amu. Ions were selected for use in selected ion monitoring (SIM) mode with an isolation width of 6.0 amu. During method development, two types of derivative were compared: methoxyamine-trimethyl silyl imidazoles (MO-TMSI) (Schweizer *et al.*, 2004), prepared using methoxyamine HCl (2%) with TMSI; and trimethyl silyl (TMS) derivatives, prepared using either bis-(trimethyl silyl)-tri fluoroacetamide (BSTFA) (Endo *et al.*, 2008; Prunet *et al.*, 2006), or trimethylchlorosilane (TMCS) (Dzeletovic *et al.*, 1995b; Larsson *et al.*, 2007). The latter two methods were found most robust in derivatisation of oxysterols in extracts generated by bioactivity assays.



### 3.3.8. Data Analysis Of Kinetic Parameters

All data obtained from HPLC-UV detection were quantified as described in section 2.12.1.5, and quantitation of all GC/MS data was performed as in section 2.13.4.

The velocity of product generation was calculated using the Michaelis-Menten equation (1) and a double reciprocal plot (2) of  $1/V$  vs  $1/[S]$ . Kinetic parameters of  $V_{max}$  and  $K_m$  were calculated using Graph Pad Prism v5.0.

**Equation (1):**  $V = V_{max}[S] / (K_m + [S])$

For a single-substrate enzyme reaction that follows Michaelis-Menten kinetics, equation 1 describes enzyme velocity as a function of time.  $V$  – velocity (rate) of the reaction,  $[S]$  – concentration of substrate,  $V_{max}$  – maximum velocity and  $K_m$  – Michaelis-Menten constant for the reaction.

$V_{max}$  is a measure of how fast the enzyme can go at full speed.  $K_m$  is a measure of how much substrate is required for the enzyme to get to full speed.  $K_m$  is the substrate concentration at half the limiting velocity (When  $[S] = K_m$ , then  $V = \frac{1}{2} V_{max}$ ). The ratio of  $V_{max}/K_m$  measures the efficiency of the enzyme, which is ultimately limited by the rate of diffusion of the substrate to the enzyme.

A Lineweaver-Burke double reciprocal plot (equation 2) of enzyme kinetics provides a linear representation of (1).

**Equation (2):**  $1/V = ((K_m/V_{max}) * 1/[S]) + 1/V_{max}$

### 3.3.9. Statistical Analysis

All data are mean  $\pm$  standard error of the mean (SEM). For m11 $\beta$ HSD1 purified recombinant protein, the means obtained from three individual experiments were calculated. All experiments using animal tissues were carried out using tissues from 6 (hepatic microsomes) to 14 (aortae) animals. Each assay was performed in duplicate or triplicate as indicated in Methods and final values of the mean per animal were taken for analysis. Statistical significance was determined using unpaired Student's t-test (section 2.14).

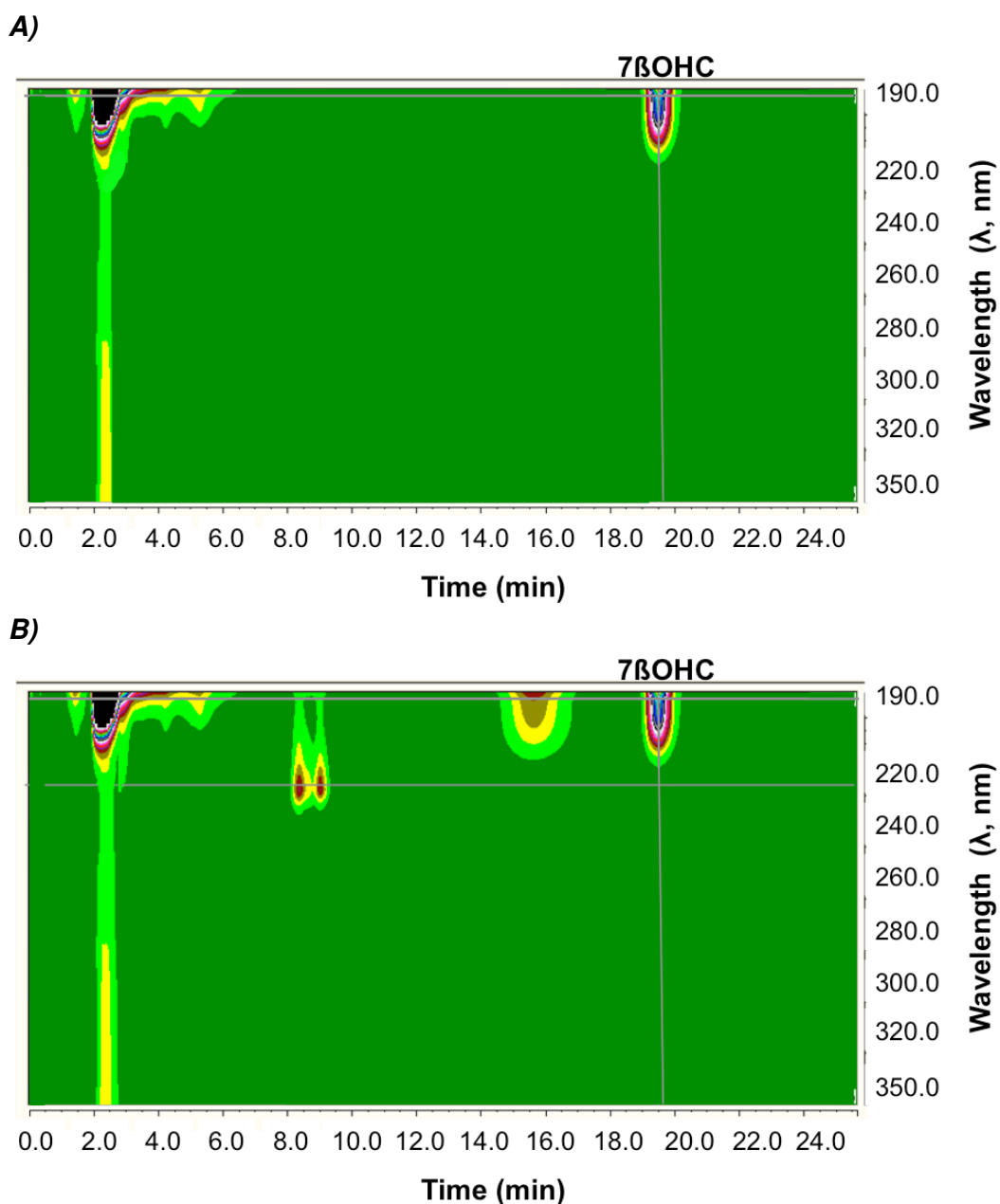
## 3.4. Results

### 3.4.1. Method Development

Prior to commencing analysis of oxysterols, several aspects of methodology were optimized, as follows.

#### 3.4.1.1. *Handling Of Oxysterol Solutions*

All oxysterol solutions were handled and stored under argon and prepared using BHT (50 µg/ml in ethanol) as this prevented oxidation of solutions and further peroxide formation. The stability of each solution was initially monitored weekly by HPLC-UV photodiode-array analysis (section 2.12) and examples of such scans of solutions of 7 $\beta$ OHC, with and without formation of peroxides, are given in Figure 3.4 – 1. By monitoring stability it was established that argon should be flushed into the vial of solution every time it was opened, and solutions should be discarded if opened more than 3 times. During assay preparation, to avoid thermal degradation and oxidation, oxysterol solutions were kept on dry ice, in a polystyrene box.



**Figure 3.4-1 HPLC-UV 3-D scan of 7 $\beta$ OHC plotted in a 2D orientation.**

Photodiode-array scans of oxysterol solutions were performed using HPLC-UV analysis. The spectrum of 7 $\beta$ OHC is plotted in 2-D orientation as elution time from the HPLC column ( $x$ -axis) vs. wavelength ( $y$ -axis). **A)** A single peak was detected in a freshly prepared solution of 7 $\beta$ OHC (195 nm,  $R_t$ 19min), whereas **B)** the effect of storage on the stability of 7 $\beta$ OHC was demonstrated by the formation of oxidation products, indicated by a non-identified peak (N.I. 195 nm,  $R_t$ 14.8min) and peroxides (220 nm,  $R_t$ 8.5min) (Caboni *et al.*, 1997), in addition to the original 7 $\beta$ OHC peak (195 nm,  $R_t$ 19 min). The peaks in  $R_t$  0=6.0 minut represent the solvent front.  $R_t$ , Retention time; nm, nanometers; min, minutes.

### 3.4.1.2. **Recovery Of Steroids And Oxysterols From Buffers**

The efficiency of steroid extraction from Krebs buffer was tested prior to starting the experiments and is reported in Table 3 - 1, showing maximal extraction efficiency with ethyl acetate.

**Table 3-1 Extraction of steroids into solvents from Krebs buffer.**

<b>Solvent</b>	<b>Percentage Recovery (%)</b>
<b>Ethyl acetate</b>	90
<b>DCM</b>	68
<b>Hexane</b>	7
<b>Petroleum ether</b>	4

Glucocorticoids were extracted from Krebs buffer using the solvents listed. Extracted samples (n=3) were dried, dissolved in mobile phase and analysed by HPLC. The percentage recovery was calculated by expressing the peak area of the extracted sample over the peak area of the un-extracted sample. The most efficient recovery for glucocorticoids was achieved with ethyl acetate. DCM, dichloromethane.

All reactions for oxysterol assays were performed in phosphate buffer (see section 3.3.2) since the reaction occurred at a faster rate compared with that in Krebs buffer of the same pH (possibly due to the absence of salts in the phosphate buffer (Song *et al.*, 1998)). The efficiency of oxysterol extraction into solvents from phosphate buffer was tested as reported (Table 3 - 2). Maximal extraction efficiency (> 90%) was obtained with petroleum ether. However, following incubation (37 °C, air) the recovery reduced (60-80%), probably due to instability of oxysterols. To avoid thermal degradation during sample processing, solvent was always evaporated under argon and at RT rather than at 60 °C.

**Table 3-2 Extraction of oxysterols into solvents from phosphate buffer.**

<b>Solvent</b>	<b>Percentage Recovery (%)</b>
<b>Petroleum ether</b>	90
<b>Petroleum ether : DCM (1:1)</b>	77
<b>Ethyl acetate</b>	68
<b>DCM</b>	51
<b>Hexane</b>	47

Oxysterols were extracted from phosphate buffer using the solvents listed. Extracted samples (n=3) were evaporated under argon, dissolved in mobile phase and analysed by HPLC. The percentage recovery was calculated by expressing the peak area of extracted sample over the peak area of the un-extracted sample. The most efficient recovery for oxysterols was achieved with petroleum ether. The range of solvents was selected based on personal communication and published references (Andrew, 2005; Hult *et al.*, 2004). DCM, dichloromethane.

#### **3.4.1.3. Recovery Of Oxysterols From DMEM And Cells**

Three separate solvent mixtures were compared to permit extraction of oxysterols from DMEM or Ham's F12, based on published references:

- (1) Hexane : 2-propanol (60 : 40) (Freeman, 2005)
- (2) Chloroform : methanol (2 : 1) (Siems *et al.*, 2005) and
- (3) Diethylether : hexane (3 : 2) (Endo *et al.*, 2008)

Extraction of oxysterols from DMEM could be performed using either mixture No (1) or No (2), but using mix No (3), the phase separation did not occur. With mixture No (1) phase separation was achieved faster (1000 g, 4°C, 5 min) whereas longer centrifugal step was required using mix No (2) (1000 g, 4°C, 20 min). To shorten the time of extraction and reduce sample exposure to the air, oxysterols were extracted using mix No (1), hexane : 2-propanol (60:40).

Extraction of oxysterols from Ham's F12 medium could only be performed using Mix No (2), as phase partition was not achieved with any other solvent mixture.

For the extraction of oxysterols from lysed cells, phase separation was only achieved using methanol : hexane (1:4) yielding ~95% recovery when PBS with EDTA was added (section 2.6.3.2) as previously described (Brown *et al.*, 1997).

Subsequent analysis of extracts by GC/MS (section 2.13) suggested that all of the above selected solvent mixtures permitted detection of 7-oxysterols (sensitivity 5 pmol/ $\mu$ l on column).

#### **3.4.1.4. Derivatisation Of Oxysterols For Detection By GC/MS**

The most widely-used reaction for derivatisation of oxysterols prior to GC/MS analysis is silylation. In this reaction a hydrogen atom is replaced by an alkylsilyl group (most often trimethylsilyl, TMS), which further prevents interaction with the stationary phase of the gas chromatographic column. TMS derivatives of oxysterols were more volatile, less polar and more thermally-stable than their parent compounds. Thus they yielded a lower limit of detection, due to improved peak intensity and symmetry (Blau and Halket, 1993; Knapp, 1979). Various conditions were tested for the silylation reactions using reagents already described (section 3.3.7.2):

##### *1) Incubation time*

During pilot work, it was noticed that a prolonged, overnight derivatisation resulted in ~ 60% loss in abundance of derivatised 7-oxysterols, whereas greater abundance (> 75%) of 7-oxysterols was obtained using derivatisation times between 30 min - 3h.

## *2) Temperatures (RT – 100 °C)*

Heating was required to facilitate the reaction (45 – 60°C). Peak height and shape upon analysis were maintained when samples were incubated at these temperatures for up to three hours. A shorter incubation time (30 min) and higher temperature (60°C) were chosen for analysis.

## *3) Sample purification*

The use of a size exclusion column of Lipidex-5000 (Packard, Pangbourne, UK) to purify the samples by removing excess derivatisation reagents resulted in irreproducible peak heights. Consequently, this step was omitted from further analyses.

## *4) The use of silanised Reacti vials*

This step to deactivate the surface of the glass reaction vials contributed further to improved abundance and stability of derivatised compounds and, therefore, silanised vials were always used for sample processing.

## *5) The use of catalyst (pyridine).*

Pyridine (1 %) was added for catalysis and gave improved peak symmetry when combined with either of the TMS derivatives.

## *6) Silylation*

Care was taken to minimize exposure of derivitising reagents to air and moisture. Samples being processed were kept under argon at all times, while stored or dried in the hot block. Repeated use of TMSI demonstrated that the product formed yielded poor peak symmetry and reproducibility, but this method was used initially for confirmation of HPLC assays until further development could be performed using alternative silylation reagents. A mixture of Bis(trimethyl)silyltrifluoroacetamide (BSTFA) and Trichloromethylsilazine (TMCS) (4:1) resulted in products yielding the same peak shape and abundance as BSTFA alone, however, this method was more costly.

The yields of derivative formed using BSTFA with pyridine (1%) versus pyridine: Hexamethyldisilazine (HMDS) : Trichloromethylsilazine (TMCS) (3:2:1, v/v/v) were compared (section 2.13.2) in combination with the following oven programs

- 1) Modified method from the literature: initial temp: 180°C (1min), Ramp 1: 35°C/min until 270°C (1min), Ramp 2: 4°C/min until 300°C and maintained for 12 min (Dzeletovic et al., 1995b; Larsson et al., 2007).
- 2) In-house method: initial temp: 165°C (1min), Ramp 1: 20°C/min until 300°C (1min), Ramp 2: 5°C/min until 330°C (8 min), Ramp 3: 10°C/min until 250°C (1 min).

The initial temperature was maintained around 174°C as this is the boiling temperature of decane, in which the derivatised samples were finally dissolved.

Overall peak symmetry was improved utilising BSTFA/pyridine in particular in combination with Method (2). However the use of Method (1) resulted in a 40% better abundance of derivatised oxysterols prepared by the pyridine : HMDS : TMCS mixture. The latter derivatisation method was ultimately proven to be more sensitive for detection of trace amounts of oxysterols in the plasma and other biological tissues compared with BSTFA/pyridine. However, the BSTFA/pyridine derivatisation method was used successfully at early stages for analysis of dried residues from tissue culture experiments or samples generated for confirmation of the HPLC results.

### 3.4.2. Substrate Modelling

3-D modelling of 7-oxysterols and docking of these structures into the enzyme active site of 11 $\beta$ HSD1 was performed as described in sections 3.4.2.2 and 3.4.2.3, prior to *in vitro* assessment of the substrate preference of 11 $\beta$ HSD1.

#### 3.4.2.1. DNA sequence alignment of 11 $\beta$ HSD1 Enzymes

Prior to substrate modelling alignment of the known amino-acid sequences of 11 $\beta$ HSD1 was performed to highlight the differences between the active sites of 11 $\beta$ HSD1 from three different species, human, murine and hamster. The comparison



of DNA sequences revealed good similarity of the enzyme structures ~78% (Figure 3.4. – 2) as shown before (Arampatzis *et al.*, 2005; Hult *et al.*, 2004).

#### 3.4.2.2. **Overlay of Enzyme Structure**

The C-terminal part of 11 $\beta$ HSD1 contains active site motif with the catalytically important sequence which is crucial for catalysis and proton transfer between substrate and cofactor: Asn<sup>143</sup> – Ser<sup>170</sup> – Tyr<sup>183</sup> – Lys<sup>187</sup> (N-S-Y-K), also referred to as catalytic tetrad (Filling *et al.*, 2002), as described in section 1.2.1.1. The presence of NADP<sup>+</sup> within the crystal structure, in particular, allows *in silico* modelling of physiological binding by superimposing the structures of human (2BEL) and murine (1Y5R) structures of 11 $\beta$ HSD1 (Figure 3.4 – 3A). The active sites showed close similarity in orientation of the murine and human active sites as demonstrated in Figure 3.4 – 3A. The 3-D structures were subsequently used to simulate binding by 7 $\alpha$ OHC, 7 $\beta$ OHC or 7-KC.

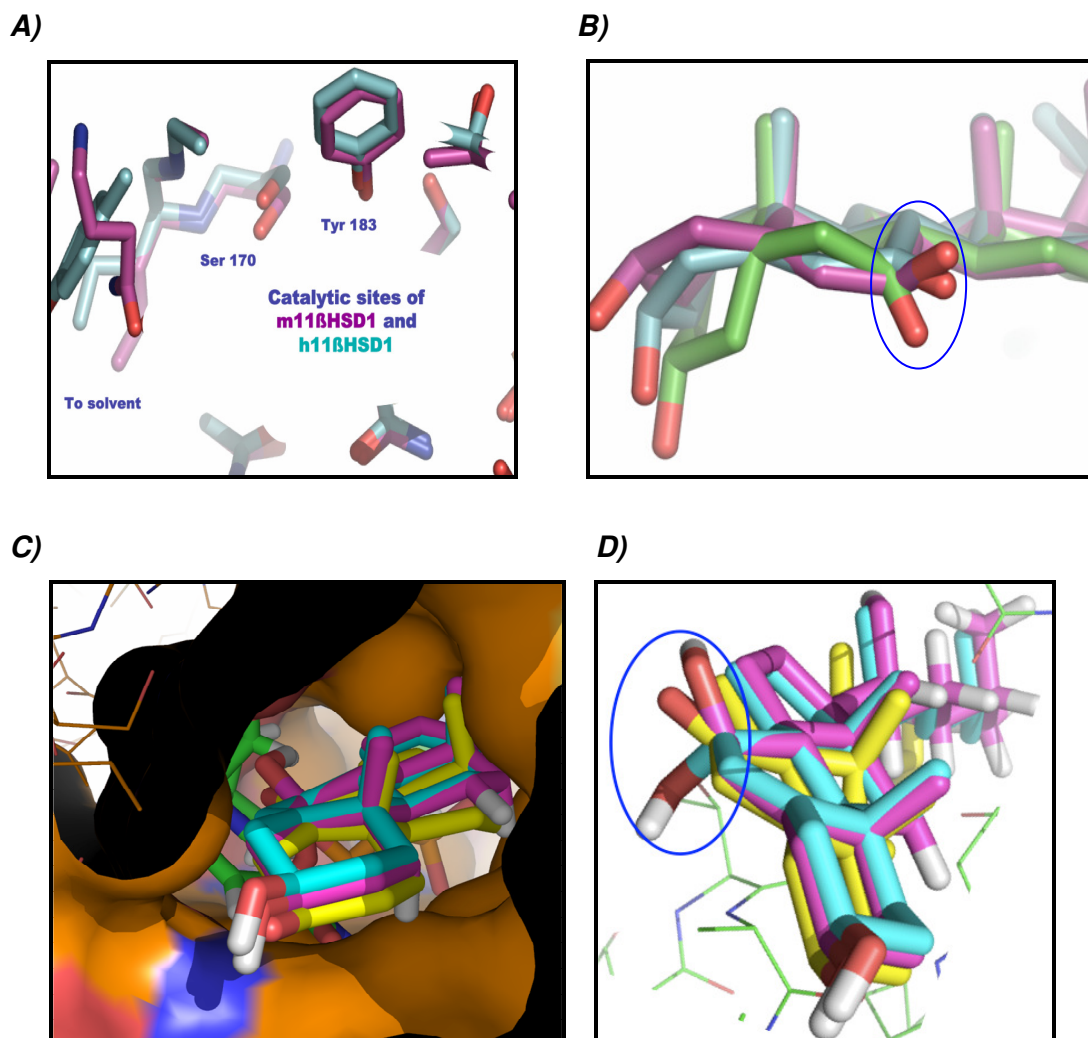
#### 3.4.2.3. **Substrate Binding To Active Site**

In order to establish orientation of the oxygenated residues at the C7 position, the structure of 7 $\alpha$ HSD in complex with 7-oxoglycochenodeoxycholic acid (1FMC) was used. When the active sites of 7 $\alpha$ HSD (1FMC) and m11 $\beta$ HSD1 (1Y5R) were overlaid, the tyrosine residue of 1FMC and the 7 $\alpha$ -hydroxyl group of the ligand superimposed with the tyrosine of 1Y5R and the 11-hydroxyl group of corticosterone. Therefore, the 3-D structure of the 7 $\alpha$ OHC was drawn using computer software, to resemble the 3-D orientation of 7-oxoglycochenodeoxycholic acid (Figure 3.4 – 3B). Subsequently, the steric positions of the 7 $\beta$ -hydroxyl and 7-keto groups of 7 $\beta$ OHC and 7KC, respectively were orientated in relation to 7 $\alpha$ HSD. All three structures of 7-oxysterols were fitted in the active site based on the energy maps created as described in the methods. This resulted in 10 clusters formation for each small molecule binding to the active site. The “middle” conformation was used, which resulted with minimum energy of predicted interaction.



A closer view of the orientation of the functional group at the C7 position of the three 7-oxysterols is shown Figure 3.4 – 3C-D.

Modelling of either 7-KC or 7 $\beta$ OHC with h11 $\beta$ HSD1 showed that Ser<sup>170</sup> and Tyr<sup>183</sup> were 2.95 Å from either the C7-ketone or C7-hydroxyl residue and the C4 of the nicotinamide ring was 2.40 Å and 2.95 Å away from Tyr<sup>183</sup>, respectively, compatible with favourable bond lengths for reactions to occur.



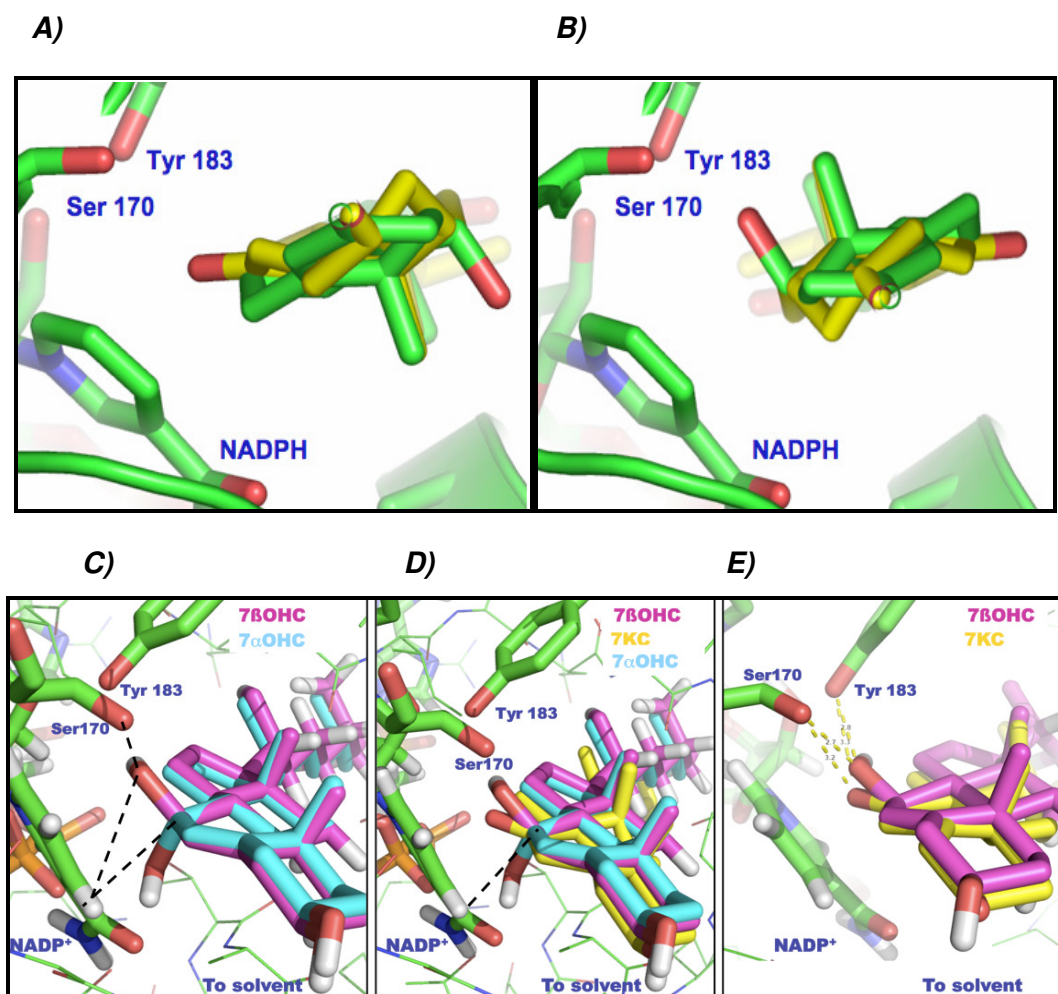
**Figure 3.4-3** *In silico* modeling of 7-oxysterols and their binding to murine and human 11 $\beta$ HSD1 structures

**A)** *In silico* models of murine (m11 $\beta$ HSD1, purple) and human (h11 $\beta$ HSD1, teal) enzyme structures overlaid; The active site residues in the murine and human enzyme may be superimposed very closely. **B)** 3-D structures of 7 $\beta$ OHC (pink) and 7-KC (blue) were compared with that of 7-oxoglycochenodeoxycholic acid (green) by overlaying the molecules to establish special orientation. **C)** Structure of murine 11 $\beta$ HSD1 with surface of the protein and overlaid 7-oxysterol substrates, 7-KC (yellow), 7 $\beta$ OHC (pink) and 7 $\alpha$ OHC (turquoise). **D)**

The overlaid 3-D structures of 7-oxysterols - 7-KC (yellow), 7 $\beta$ OHC (pink) and 7 $\alpha$ OHC (turquoise) which are substrates for 11 $\beta$ HSD1, showing the different stereochemistry at the C7 position.

To further confirm that the structures of 7-oxysterols have the orientation of a substrate, rather than of an inhibitor, the 7-oxysterol models were also compared with the 3-D orientation of glucocorticoids and carbenoxolone (inhibitor) in 2BEL structure. Binding of an inhibitor (carbenoxolone) was such that it positions itself within the rings of corticosterone, to prevent its further binding to the active site. The orientation of 7-oxysterols within the active site, however, resembles that of the substrate and favours catalysis. The 3-D analysis with m11 $\beta$ HSD1 indicated that, during catalysis, the A ring of 7-oxysterols is orientated towards the interior of the enzyme, as seen in Figure 3.4 – 4A-B, whereas with corticosterone 11 $\beta$ HSD1 interacts with the D ring. As such the orientation of the C7 group in 7-KC assumes the same position as that of the C11 group in corticosterone rotated by 180°. Therefore these two groups possibly locate within a similar binding site, corresponding to the murine or human 11 $\beta$ HSD1 active site (Wu *et al.*, 2006). The differences in the ring orientation of these two classes of substrates might possibly influence the kinetic of 11 $\beta$ HSD1.

Figure 3.4 – 4C-E shows the interaction of Ser<sup>170</sup> and Tyr<sup>183</sup> with the 7-oxysterol substrates and Tyr<sup>183</sup> with the NADPH. These are the highly conserved interaction in SDR proteins (Jornvall *et al.*, 1995).



**Figure 3.4-4** *In silico* models of glucocorticoids and oxysterols binding as substrates to m11 $\beta$ HSD1

**A)** 11-Dehydrocorticosterone (green) and 7-ketocholesterol (7-KC, yellow) were superimposed within the active site of m11 $\beta$ HSD1. Superimposed substrates are orientated with the 7-keto group of 7-KC facing the active site. The catalytic tetrad of the m11 $\beta$ HSD1 (1Y5R) is comprising Asn, Ser, Tyr and Lys residues. **B)** In contrast, rotational symmetry of **A)** by 180° results in the 11-keto group of 11-dehydrocorticosterone facing the active site. **C)** Interaction of the active site residues with the superimposed structures of 7 $\alpha$ OHC (turquoise) and 7 $\beta$ OHC (pink). **D)** Interaction of m11 $\beta$ HSD1 with overlaid 7-oxysterol molecules (7 $\alpha$ OHC, 7 $\beta$ OHC and 7-KC). **E)** A closer view of the interactions between 7 $\beta$ OHC (pink) and 7-KC (yellow) with the key residues of the active site and NADP<sup>+</sup>.

Critical distance of hydrogen bonding between 7-oxysterols and murine 11 $\beta$ HSD1 molecule are shown in Table 3 – 3.

Upon modelling of 7-KC with m11 $\beta$ HSD1 (Fig 3.4 – 4D-E), Ser<sup>170</sup> and Tyr<sup>183</sup> were 3.2Å and 3.3Å respectively from the C7 ketone of 7-KC. Carbon atom on C7 ketone was 3.0Å from the nicotinamide C4. The planar structure of 7-KC suggested favourable catalysis with all three hydrogens on Tyr<sup>183</sup>, Ser<sup>170</sup> or NADP<sup>+</sup> (see Table 3 – 3).

Upon modelling of 7 $\beta$ OHC into the active site of m11 $\beta$ HSD1 (Fig 3.4 – 4A-C) Ser<sup>170</sup> and Tyr<sup>183</sup> were 2.8 Å and 2.7 Å respectively from the C7 hydroxyl of 7 $\beta$ OHC. In the same model, carbon atom on C7 hydroxyl was 3.0 Å from the nicotinamide C4 (Table 3 – 3). The chair orientation assumed by 7 $\beta$ OHC provided a favourable orientation for catalysis within the m11 $\beta$ HSD1.

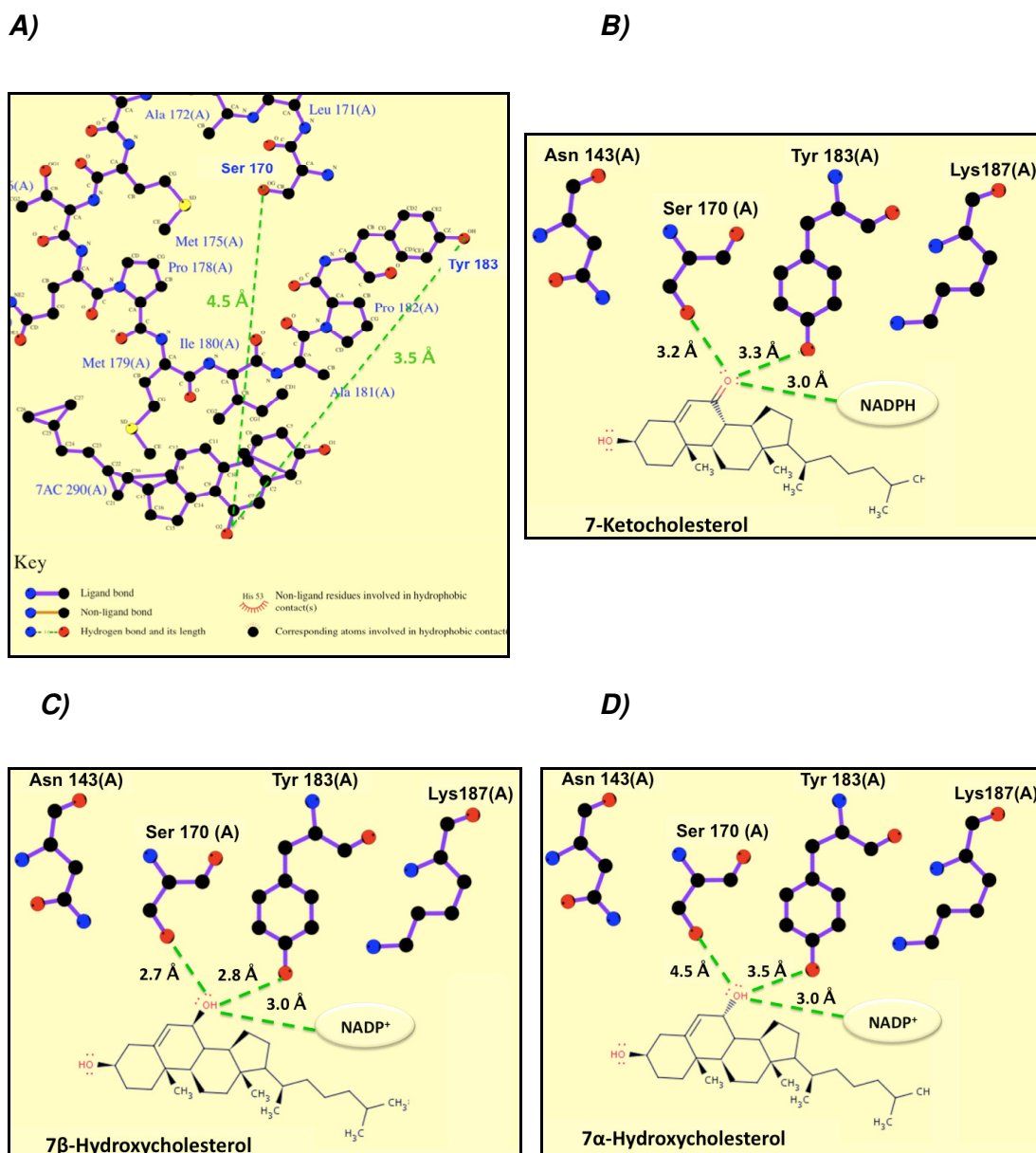
Upon modelling of 7 $\alpha$ OHC into the active site of m11 $\beta$ HSD1 (Fig 3.4 – 4C-D), Ser<sup>170</sup> and Tyr<sup>183</sup> were 4.5Å and 4.8Å respectively from the C7 hydroxyl of 7 $\alpha$ OHC. Carbon atom on C7 hydroxyl was 3.0 Å from the nicotinamide C4. In contrast to 7 $\beta$ OHC, the 90°C “tilt” in orientation of the C7-hydroxyl group in 7 $\alpha$ OHC, showed that 7 $\alpha$ OHC was less tightly bound within the active site (Figure 3.4 – 4C-D), as it was positioned too far away for efficient interaction (Table 3 – 3).

Overall the results of *in silico* docking of substrates into the active site of m11 $\beta$ HSD1 suggested that 7 $\beta$ OHC and 7-KC, but not 7 $\alpha$ OHC are putative substrates for this enzyme. Further distances obtained in this experiment are tabulated below. Further 2-D LigPlot representation of substrate-protein interaction is detailed as shown in Figure 3.4 – 5.

**Table 3-3 Critical distances of hydrogen bonding between substrates for 11 $\beta$ HSD1 and enzyme active site residues.**

<b>Distance (amstrongs Å)</b>	<b>7aOHC</b>	<b>7BOHC</b>	<b>7KC</b>
Oxygen on C7 to O-gamma atom of Ser <sup>170</sup> side chain	4.5	2.7	3.2
Carbon C7 to O-gamma atom of Ser <sup>170</sup> side chain	3.5	3.5	3.5
Oxygen on C7 to Tyr <sup>183</sup> side chain	4.8	2.8	3.3
Carbon C7 to Tyr <sup>183</sup> side chain	4.1	4.1	4.1
Carbon C7 on 7-oxysterol to C4 of NADPH	3	3	3
Oxygen on C7 of 7-oxysterol to C4 of NADPH	3	3	2.3
Oxygen on Tyr <sup>183</sup> to carbon C4 on NADPH	5.1	5.1	5.1
Lys <sup>187</sup> side chain to each ribose 2'OH on NADPH	3.2	3.2	3.2

The numbers represent the distances (in angstroms, Å) of functional groups on carbon C7 of 7-oxysterols to a key residue (serine, Ser<sup>170</sup>; tyrosine, Tyr<sup>183</sup> and lysine, Lys<sup>187</sup>) in the active site of murine 11 $\beta$ HSD1 enzyme (PDB structure: 1Y5R) as obtained using Pymol software.



**Figure 3.4-5 Interaction of 7-oxysterols with murine 11βHSD1 protein.**

**A)** 2-D LigPlot representation of murine 11βHSD1 (m11βHSD1, PDB structure 1Y5R) enzyme catalytic residues interacting with 7α-hydroxycholesterol. 2-D LigPlot representation of active site catalytic tetrad interacting with **B)** 7-ketocholesterol, **C)** 7β-hydroxycholesterol and **D)** 7α-hydroxycholesterol orientation of which (**B-D**) is represented using MarvinView software (ChemAxon). Overall the results of *in silico* docking of substrates into the m11βHSD1 active site suggested that 7βOHC and 7-KC, but not 7αOHC, are putative substrates for this enzyme. Key distances, from the active C7 oxygen on 7-oxysterols to serine (Ser170) and tyrosine (Tyr183) residue and to cofactor (NADP or NADPH) is given in angstroms (Å) as obtained using Pymol software. Lys187 (lysine), Asn143 (asparagine).

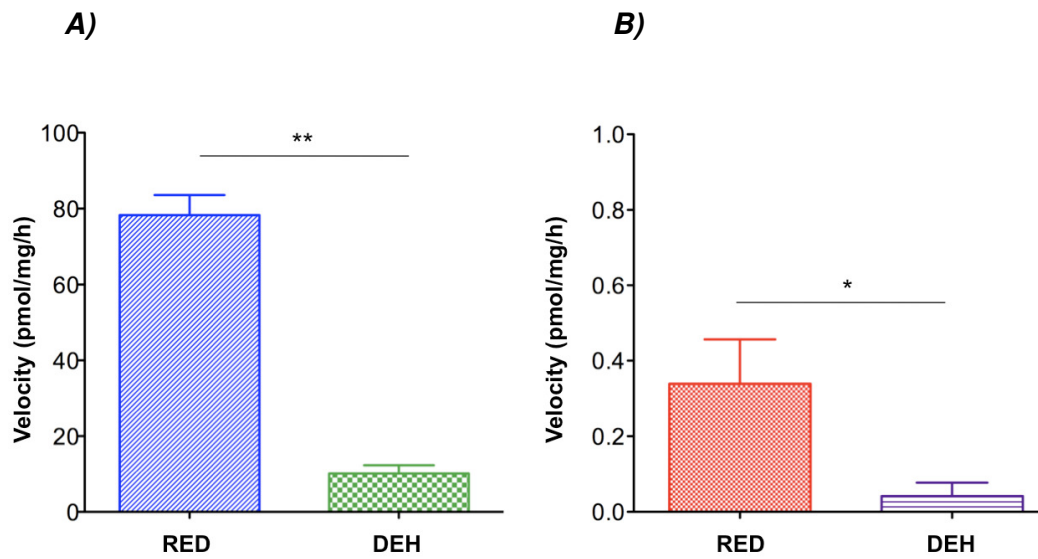


### **3.4.3. *In Vitro* Assessment Of 11 $\beta$ HSD Activity**

Purified recombinant m11 $\beta$ HSD1, or protein present in HEK293 cells were used to characterise the reduction and dehydrogenation of 7-oxysterols. Further sources of m11 $\beta$ HSD1, (lysed cells, hepatic microsomes and aortic rings) were also used. In all models, the reaction direction was co-factor-dependent and this was controlled, where possible, by modifying assay conditions. In intact cells, the reaction was driven by the endogenous cofactor.

#### **3.4.3.1. *Purified Recombinant Protein***

Both 7-KC and 7 $\beta$ OHC were metabolised by m11 $\beta$ HSD1 to give the resultant products, 7 $\beta$ OHC and 7-KC, respectively (Figure 3.4 – 5), and reduction proceeded more rapidly than dehydrogenation. Metabolism of glucocorticoids, however, proceeded at a much faster rate and again showed the expected pattern of predominant reduction and slower dehydrogenation. Conversion to 7-KC was not observed when 7 $\alpha$ OHC was used as a substrate, nor was 7 $\alpha$ OHC generated as a product upon reduction of 7-KC by 11 $\beta$ HSD1. The identities of products were confirmed by two different chromatographic methods (HPLC-UV and GC/MS).

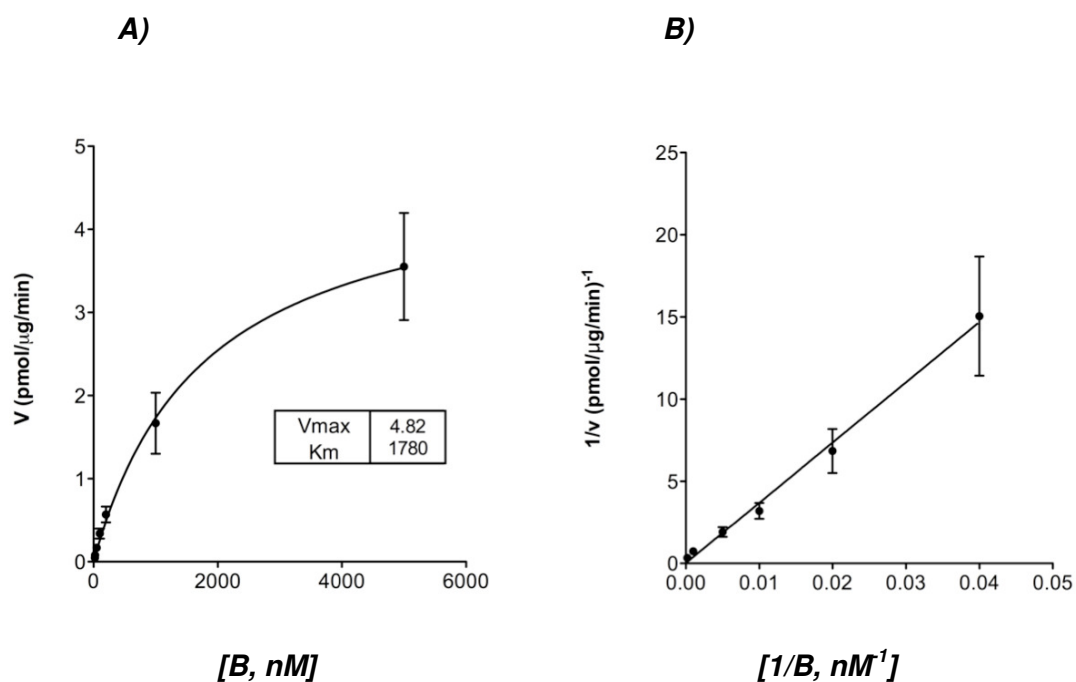


**Figure 3.4-6 Metabolism of steroids and oxysterols by m11βHSD1 (recombinant protein).**

**A)** Metabolism of steroids by m11βHSD1. Velocities of reduction (RED) of 11-dehydrocorticosterone (A) to corticosterone (B), (blue) and dehydrogenation (DEH) of B → A (green) were measured. Reduction by m11βHSD1 was predominant over dehydrogenation. **B)** Metabolism of 7-oxysterols by m11βHSD1. Velocities of reduction (RED) of 7-ketocholesterol (7-KC) to 7β-hydroxycholesterol (7βOHC) (red) and dehydrogenation (DEH) of 7βOHC → 7-KC (purple) were measured. Reduction of 7-KC by m11βHSD1 was predominant over dehydrogenation of 7βOHC. All data are mean ± SEM, compared using Student's t-test, n=6, \*p<0.05, \*\*p<0.01.

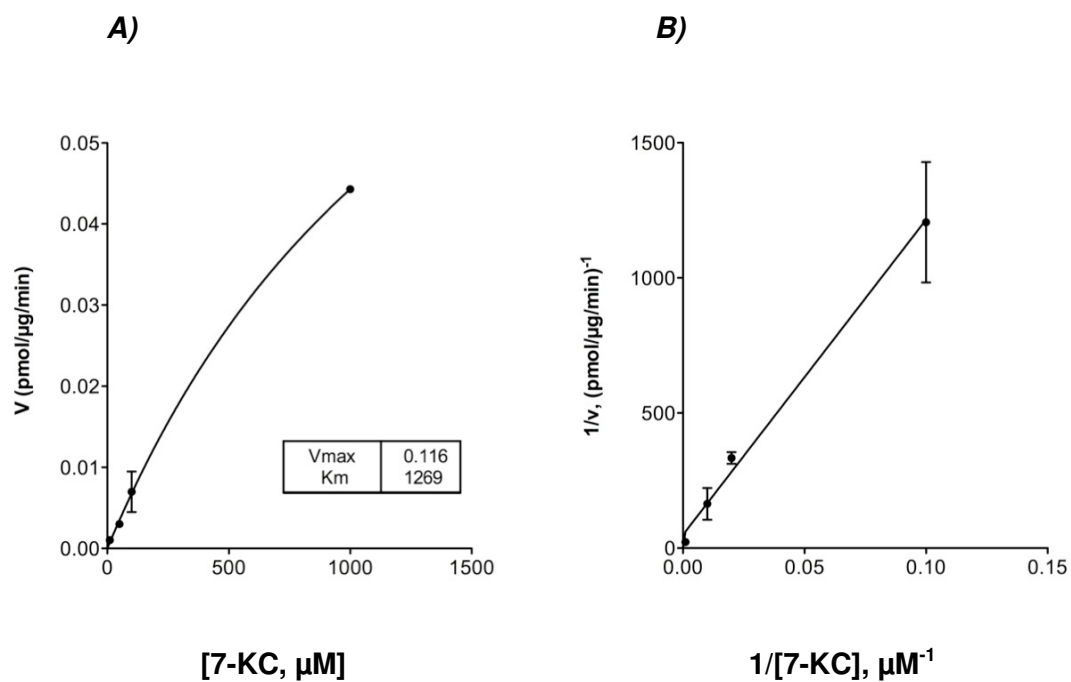
The presence of cofactor was necessary for these reactions to proceed. Metabolism of 7 $\beta$ OHC was observed in the presence of either NAD<sup>+</sup> or NADP<sup>+</sup> (velocity  $0.022 \pm 0.010$  vs.  $0.035 \pm 0.020$  pmol/ $\mu$ g/min respectively, n=3), while reduction utilized NADPH cofactor only.

In addition to assessing the ability to convert specific substrates, reaction kinetics were determined ( $V_{max}$  and  $K_m$ ) by generating a Michaelis-Menten plot and a Lineweaver-Burk transformation of it (exemplar Figure 3.4 – 7 and –8). Kinetic constants were compared between all substrates (7-KC, 7 $\beta$ OHC, A and B) as given in Table 3-4. 7 $\beta$ OHC binds better to 11 $\beta$ HSD1 compared with 7-KC as indicated by a lower  $K_m$  value. On the other hand, the enzyme preference for glucocorticoids over 7-oxysterols is demonstrated by the lower  $K_m$  values for the former, suggesting tighter binding of conventional substrates. Maximal velocity ( $V_{max}$ ) of conversion of glucocorticoids by 11 $\beta$ HSD1 showed that these reactions are approximately 40 times faster than that of 7-oxysterols (Figure 3.4 – 7A & – 8A). Efficiency of 11 $\beta$ HSD1 conversion of substrates was calculated by obtaining the ratio of  $V_{max}/K_m$  and these measurements also suggest that murine recombinant 11 $\beta$ HSD1 is converting 11-dehydrocorticosterone more efficiently than oxysterols. Furthermore, recombinant 11 $\beta$ HSD1 converts 11-dehydrocorticosterone over 30-times more efficiently than corticosterone; and 7-ketocholesterol almost 3-times more efficiently than 7 $\beta$ -hydroxycholesterol.



**Figure 3.4-7 Enzyme kinetics – Metabolism of glucocorticoids by m11 $\beta$ HSD1**

**A)** Michaelis-Menten Plot of velocities of conversion of corticosterone to 11-dehydrocorticosterone (dehydrogenation) by recombinant m11 $\beta$ HSD1;  $V_{max}$  (pmol/ $\mu$ g/min) and  $K_m$  (nM) were calculated. **B)** Lineweaver-Burk Plot, representing the linearised plot of **(A)**. All data are mean  $\pm$  SEM,  $n=3$ , in duplicate.



**Figure 3.4-8 Enzyme kinetics – metabolism of 7-oxysterols by 11βHSD1**

**A)** Michaelis-Menten Plot of the velocities of conversion of 7-KC to 7βOHC (reduction) by recombinant m11βHSD1 protein.  $K_m$  (μM) and  $V_{\text{max}}$  (pmol/μg/min) were calculated **B)** Lineweaver-Burk linear plot of **(A)**. All data are mean  $\pm$  SEM,  $n=3$ , in duplicate.

**Table 3-4 Summary of kinetic constants for reactions catalysed by recombinant m11 $\beta$ HSD1.**

m11HSD1 (n=3)	K <sub>m</sub> ( $\mu$ M)	V <sub>max</sub> (pmol/ $\mu$ g/min)	V <sub>max</sub> /K <sub>m</sub>
<b>11-Dehydrocorticosterone</b>	0.2 $\pm$ 0.25	8.56 $\pm$ 4.06	87.18 $\pm$ 13.78
<b>Corticosterone</b>	1.78 $\pm$ 0.56	4.82 $\pm$ 0.65	2.69 $\pm$ 0.57
<b>7-Ketocholesterol</b>	1269 $\pm$ 282 *	0.116 $\pm$ 0.03	0.07 $\pm$ 0.006 *
<b>7<math>\beta</math>-Hydroxycholesterol</b>	327.6 $\pm$ 98.5 *	0.010 $\pm$ 0.001	0.03 $\pm$ 0.003

Michaelis constants (K<sub>m</sub>,  $\mu$ M) and the maximal velocities (V<sub>max</sub>, pmol/ $\mu$ g/min) were calculated for each direction of metabolism of glucocorticoids (GC) and oxysterols by murine recombinant 11 $\beta$ HSD1 (m11 $\beta$ HSD1). Lower K<sub>m</sub> values indicate that GCs are better substrates for 11 $\beta$ HSD1 than 7-oxysterols and that 7 $\beta$ OHC binds more tightly to the enzyme than 7-KC. Efficiency of 11 $\beta$ HSD1 conversion of substrates was calculated by obtaining the ratio of V<sub>max</sub>/K<sub>m</sub>,  $\mu$ l.min/ $\mu$ g for glucocorticoids and V<sub>max</sub>/K<sub>m</sub>  $\mu$ l.min/ng for 7-oxysterols. These measurements suggest that 11 $\beta$ HSD1 is converting 11-dehydrocorticosterone almost 15-times more efficiently than corticosterone; and likewise metabolizes 7-ketocholesterol 3-times more efficiently than 7 $\beta$ -hydroxycholesterol. V<sub>max</sub> of conversion of glucocorticoids by 11 $\beta$ HSD1 was approximately 40 times higher than that recorded for 7-oxysterol. All data are mean  $\pm$  SEM, compared using an unpaired Students t-test, \*p<0.05 compared with appropriate glucocorticoid, n=3, duplicate.

#### 3.4.3.2. m11 $\beta$ HSD1 In Intact And Lysed Cells

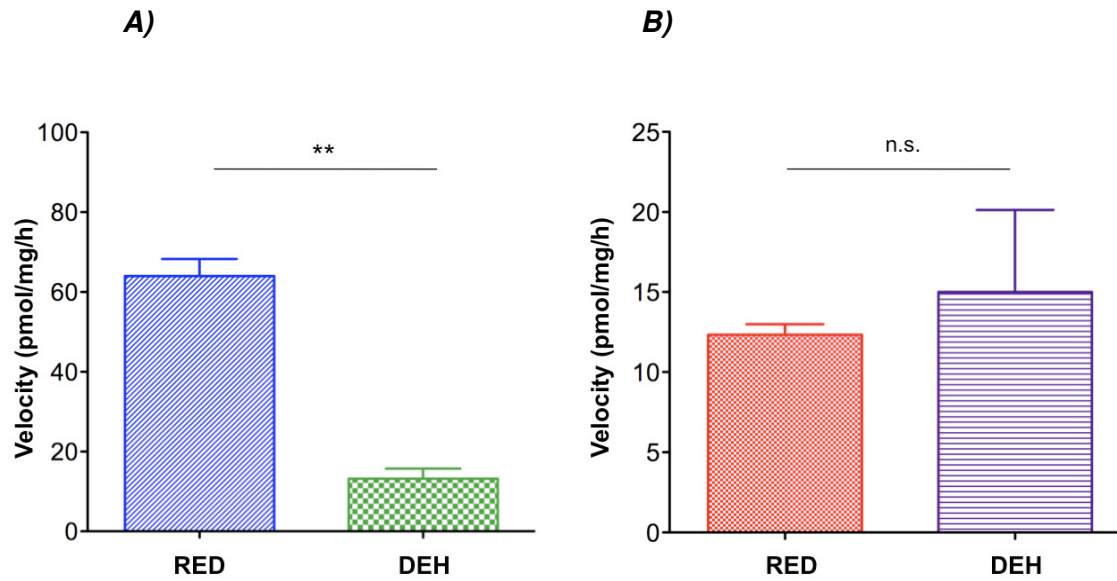
In order to simulate the intracellular environment, the velocity and favoured direction of metabolism by m11 $\beta$ HSD1 expressed, following stable transfection, in CHO or HEK293 cells was assessed. Data relating to directionality from CHO cells proved to be variable but HEK293 cells, stably transfected with mouse *HSD11B1*, proved more robust. Therefore, these transformed HEK293 cells were also used for assessment of the metabolism of oxysterol by 11 $\beta$ HSD1. Negative controls were the non-transfected cells, in which conversion of steroids or oxysterols was not detected. The

identities of assay products were confirmed by both HPLC with radio detection and GC/MS (sections 2.12.1.5 and 2.13.4).

Reduction of glucocorticoids (keto steroids) by m11 $\beta$ HSD1 proceeded more rapidly than dehydrogenation of hydroxy steroids (Figure 3.4 – 8A) and this was consistent with results obtained using recombinant enzyme (section 3.4.3.1). Measurements of inter-conversion of 7-oxysterols by m11 $\beta$ HSD1 in HEK293 cells showed that dehydrogenation and reduction proceeded at similar rates (Figure 3.4 – 8B) but these rates were slower than those measured using glucocorticoids as substrates. 7 $\alpha$ OHC was not a substrate for these reactions, as product (7-KC) was not detected upon analysis. Likewise 7 $\alpha$ OHC was not generated from 7-KC.

HEK293 cells demonstrated the ability to sequester added oxysterols and so the distribution of the added substrates following incubation was assessed and amounts of products formed were quantified in the cells and the medium. The data are expressed as a percentage of total oxysterol recovered and are presented in Figure 3.4 – 9. A large percentage of the 7-KC (~ 70%) provided for reduction was retained in the media, while only ~ 20% entered the cells. 7 $\beta$ OHC is more readily transported across the cell membrane into the cell (with only ~ 30% of the amount provided remaining in the media). The products of dehydrogenation (i.e. 7-KC) were detected in the media, whereas the product of reduction (i.e. 7 $\beta$ OHC) was mainly retained in cells.

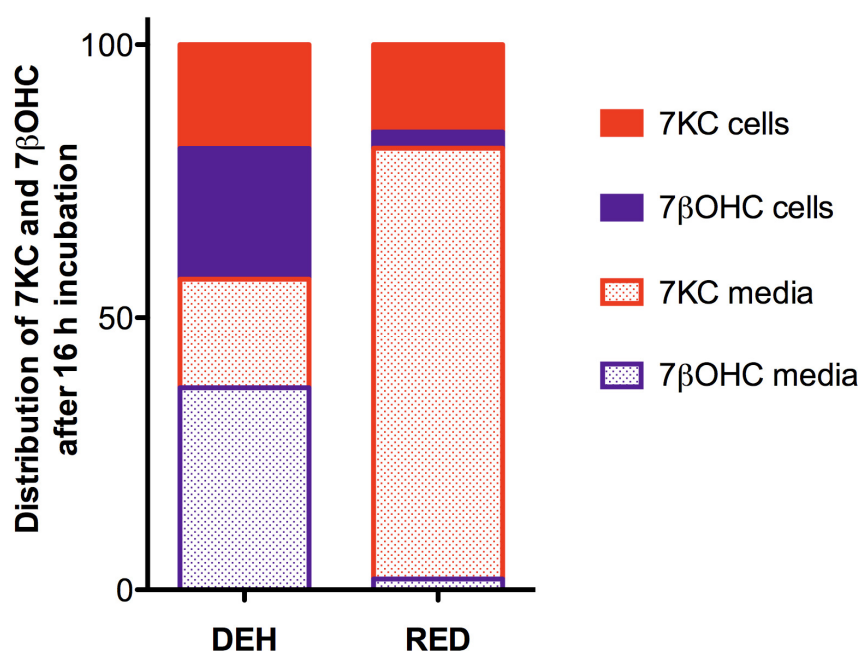
To bypass any problems with entry of 7-KC into cells, reduction of this oxysterol was also performed using m11 $\beta$ HSD1 harvested from lysed HEK293 cells. In this case the velocity of production of 7 $\beta$ OHC obtained ( $1.62 \pm 0.51$  pmol/mg/h) was slower compared with that observed in intact cells (see 3.4.3.2) but, nevertheless, faster than that observed using the recombinant protein alone (see section 3.4.3.1). Likewise, the  $K_m$  associated with the reaction was lower ( $77.7 \pm 45.1$   $\mu$ M) compared with that ascribed to recombinant m11 $\beta$ HSD1 (see Table 3 - 4) indicating tighter binding of this substrate to 11 $\beta$ HSD1 in the simulated intracellular environment.



**Figure 3.4-9 Metabolism of steroids and oxysterols by m11βHSD1 generated in HEK293 cells.**

**A)** Velocities of reduction (RED) of 11-dehydrocorticosterone (A) to corticosterone (B), (blue) and dehydrogenation (DEH) of B → A (green) were measured in intact HEK293 cells. Reduction was predominant over dehydrogenation **B)** Velocities of reduction (RED) of 7-ketocholesterol (7-KC) to 7β-hydroxycholesterol (7βOHC) (red) and dehydrogenation (DEH) of 7βOHC → 7-KC (purple) by m11βHSD1 proceeded at a similar rate. All data are mean ± SEM, compared using unpaired Student's t-test, n=6, in duplicate, \*\* p<0.01, n.s., not significant.





**Figure 3.4-10 Distribution of 7-Oxysterols between HEK293 cells and the medium following incubation.**

HEK293 cells stably transfected to produce m11 $\beta$ HSD1 were used to assess reduction (RED) of 7-ketocholesterol (7-KC) and dehydrogenation (DEH) of 7 $\beta$ -hydroxycholesterol (7 $\beta$ OHC). With each substrate added to cells for the incubation, the amounts of remaining and newly-formed oxysterol were quantified in the medium and in the cells. When 7 $\beta$ OHC was added as a substrate, 20 $\pm$ 7% of the total amount recovered accumulated in the cells, and 37 $\pm$ 6% remained in the medium. Newly formed 7-KC was detected in cells (14 $\pm$ 3% of the amount of recovered substrate) and in the medium (12 $\pm$ 4%). When 7-KC was added as a substrate, 80 $\pm$ 1% of the amount recovered was detected in the medium, and only 14 $\pm$ 4% had entered the cells. 7 $\beta$ OHC that is formed from 7-KC was detected in both media (2 $\pm$ 2% of the amount of recovered substrate) and cells (3 $\pm$ 1%). All data are mean  $\pm$  SEM, n=6, in duplicate.

### 3.4.4. *Ex Vivo* Assessment Of 11 $\beta$ HSD1 Activity

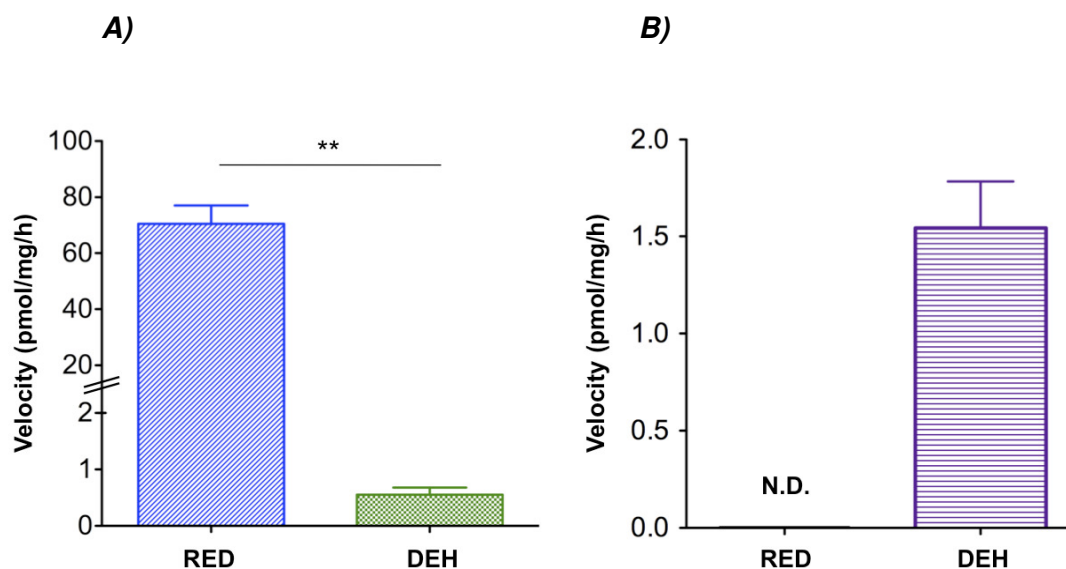
Hepatic microsomes and aortic rings were used as a source of membrane bound 11 $\beta$ HSD1 to establish the preferred direction for metabolism of 7-oxysterols *ex vivo*.

#### 3.4.4.1. 11 $\beta$ HSD1 Metabolism In Murine Hepatic Microsomes

Reduction of 11-dehydrocorticosterone to corticosterone was predominant and, although dehydrogenation of corticosterone was also observed, it proceeded at a slower rate than reduction (Figure 3.4 – 11A). Therefore the kinetics of steroid metabolism in microsomal protein from WT murine livers was consistent with those described for recombinant protein and the intact cells (see sections 3.4.2.1 and 3.4.3.1). Reactions only proceeded in the presence of cofactor and the identities of products were confirmed by HPLC-UV detection and GC/MS.

The NADP<sup>+</sup>-dependent dehydrogenation of 7 $\beta$ OHC proceeded in microsomes, as with stably transfected HEK293 cells, in the presence of cofactor but reduction of 7-KC was not observed under any conditions tested. The preference for cofactor was assessed and it was established that formation of 7-KC from 7 $\beta$ OHC could utilise either NAD<sup>+</sup> or NADP<sup>+</sup> (Maser and Netter, 1989) with similar preference (velocity  $1.25 \pm 0.20$  vs.  $1.35 \pm 0.40$  pmol/ $\mu$ g/min respectively, n=3).

Numerous manipulations were attempted to encourage the reduction of 7-KC. To address concerns over access of 7-KC to the luminal surface of microsomes, reduction of 7-KC was also assessed with the addition of the permeabilisation agent allametcine. In a separate assay, reduction of 7-KC was assessed using the NADPH generating system. Despite the addition of allametcine, or use of the NADPH generating system, the reduced product, 7 $\beta$ OHC, was not generated (Figure 3.4 – 11B). Reduction of 7-KC also did not proceed in the presence of the cofactor NADH. Following incubation, the percentage of 7-KC remaining in the incubate was close to 100% of that added (Table 3 - 5), indicating the substrate was stable and the



**Figure 3.4-11 Metabolism of steroids and oxysterols by 11 $\beta$ HSD1 in murine hepatic microsomes.**

**A)** Metabolism of steroids by 11 $\beta$ HSD1 in murine hepatic microsomes showing predominant reduction of 11-dehydrocorticosterone (blue), and slower dehydrogenation of corticosterone (green). **B)** The metabolism of 7-oxysterols in murine hepatic microsomes proceeds only by dehydrogenation (7 $\beta$ -hydroxycholesterol (7 $\beta$ OHC)  $\rightarrow$  7-ketocholesterol (7-KC), purple) and the product of reduction (7-KC  $\rightarrow$  7 $\beta$ OHC) was not detected. All data are mean  $\pm$  SEM, n=6, in duplicate, \*\*p<0.01. N.D., not detected.

explanation for lack of measurable activity was not due to degradation or alternative routes of metabolism of the substrate. Some product formation ( $2.3 \pm 0.6 \%$ ) was detected in the reduction assay upon GC/MS analysis. This was above the limit of detection (L.O.D.) and only within two fold of blank, therefore it is still within the error of the assay quantitation. In the same assay majority of the substrate was successfully recovered ( $89.6 \pm 2.2\%$ , Table 3 – 5).

As with other models tested,  $7\alpha\text{OHC}$  was not a substrate for microsomal m11 $\beta$ HSD1, nor was  $7\alpha\text{OHC}$  generated as a product of reduction of 7-KC. Again, as shown in Table 3 - 5,  $7\alpha\text{OHC}$  was stable under these conditions.

**Table 3-5 Stability of 7-oxysterols following incubation with murine hepatic microsomes.**

<b>Assay (n=6)</b>	<b>% Product</b>	<b>% Remaining substrate</b>
<b><math>7\alpha\text{OHC} \rightarrow 7\text{-KC}</math></b>	0	$98.0 \pm 1.8$
<b><math>7\beta\text{OHC} \rightarrow 7\text{-KC}</math></b>	$26.5 \pm 2.4$	$66.2 \pm 0.9$
<b><math>7\text{-KC} \rightarrow 7\beta\text{OHC}</math></b>	$2.3 \pm 0.6$	$89.6 \pm 2.2$

This table summarises the percentage of substrate remaining and product formed during incubation of 7-oxysterols with hepatic microsomes from wild type mice. 7-Oxysterols were quantified by GC/MS. All data are mean  $\pm$  SEM, n=6 in duplicate.  $7\beta\text{OHC}$ ,  $7\beta$ -hydroxycholesterol;  $7\alpha\text{OHC}$ ,  $7\alpha$ -hydroxycholesterol; 7-KC, 7-ketcholesterol.

In addition to assessing the ability of murine microsomal 11 $\beta$ HSD1 to convert specific substrates, reaction kinetics were determined ( $V_{\max}$  and  $K_m$ ) and constants obtained summarised (Table 3 - 6).  $K_m$  values for glucocorticoids acting as substrates were three orders of magnitude lower than those of 7-oxysterols, signifying their ability to bind more tightly to m11 $\beta$ HSD1.  $V_{\max}$  (Table 3 - 6) for oxidation of 7 $\beta$ OHC was three orders of magnitude lower than that for glucocorticoids (pmol/mg/min vs. pmol/ $\mu$ g/min), showing faster metabolism of glucocorticoids in comparison with 7-oxysterols. Efficiency of 11 $\beta$ HSD1 conversion of substrates was calculated by obtaining the ratio of  $V_{\max}/K_m$  ( $\mu$ l.min/ng), and these measurements suggest that hepatic 11 $\beta$ HSD1 in microsomal preparation is converting 11-dehydrocorticosterone eight time more efficiently than corticosterone. Further, both of these substrates were also metabolised more efficiently by microsomal 11 $\beta$ HSD1 compared with 7 $\beta$ -hydroxycholesterol.

#### 3.4.4.2. **11 $\beta$ HSD1 Reaction Specificity For 7-Oxysterols**

The specificity of 11 $\beta$ HSD1 to convert 7-oxysterols (7 $\beta$ OHC, 7 $\alpha$ OHC or 7-KC) was assessed using hepatic microsomes from 11 $\beta$ HSD1<sup>-/-</sup> mice. When 7 $\beta$ OHC was used as a substrate, conversion to 7-KC was not detected (whether either NAD<sup>+</sup> or NADP<sup>+</sup> were used as cofactors). Furthermore the majority of 7-KC added was successfully recovered ( $86.1 \pm 4.2$  %) from an assay utilising microsomes from 11 $\beta$ HSD1<sup>-/-</sup> mice, and was similar to that of recovery obtained using microsomes from C57Bl/6 mice. This finding implies lack of any other routes of metabolism of 7-KC under the conditions tested, supporting the hypothesis that only 11 $\beta$ HSD1 is responsible for mediating the conversion of 7 $\beta$ OHC to 7-KC in these preparations. Products were also not formed when 7 $\alpha$ OHC was added as substrate.

**Table 3-6 Summary of kinetic constants for murine microsomal 11 $\beta$ HSD1.**

<b>Murine Hepatic Microsomes</b>			
<b>Substrate</b>	<b>K<sub>m</sub> (nM)</b>	<b>V<sub>max</sub> (pmol/<math>\mu</math>g/min)</b>	<b>V<sub>max</sub>/K<sub>m</sub> (<math>\mu</math>l.min/<math>\mu</math>g)</b>
<b>11-Dehydro-corticosterone</b>	1268 $\pm$ 539	1.19 $\pm$ 0.18	83.21 $\pm$ 12.39
<b>Corticosterone</b>	4199 $\pm$ 2095	0.04 $\pm$ 0.01	10.87 $\pm$ 2.74
<b>Substrate</b>	<b>K<sub>m</sub> (<math>\mu</math>M)</b>	<b>V<sub>max</sub> (pmol/mg/min)</b>	<b>V<sub>max</sub>/K<sub>m</sub> (<math>\mu</math>l.min/ng)</b>
<b>7-Ketocholesterol</b>	n/d	n/d	n/d
<b>7<math>\beta</math>-Hydroxy-cholesterol</b>	3500 $\pm$ 326	32.6 $\pm$ 9.9	0.009 $\pm$ 0.01

The binding constants (K<sub>m</sub>) and the maximal velocities (V<sub>max</sub>) were calculated for each direction of metabolism of steroids and oxysterols by 11 $\beta$ HSD1 in murine hepatic microsomes. The preference of m11 $\beta$ HSD1 to accept glucocorticoids as substrates over 7-oxysterols was demonstrated by a K<sub>m</sub> value, which was three orders of magnitude lower. The velocities (V<sub>max</sub>) of 11 $\beta$ HSD1 oxidising 7 $\beta$ -hydroxycholesterol were three orders of magnitude lower than that of oxidation of corticosterone (pmol/mg/min vs. pmol/ $\mu$ g/min). 7-Ketocholesterol was not reduced by m11 $\beta$ HSD1. (n/d, not detected). Efficiency of 11 $\beta$ HSD1 conversion of substrates was calculated by obtaining the ratio of V<sub>max</sub>/K<sub>m</sub>,  **$\mu$ l.min/ $\mu$ g** for glucocorticoids and V<sub>max</sub>/K<sub>m</sub>  **$\mu$ l.min/ng** for 7-oxysterols. These measurements suggest that 11 $\beta$ HSD1 is converting 11-dehydrocorticosterone more efficiently than corticosterone or 7 $\beta$ -hydroxycholesterol. All data are mean  $\pm$  SEM, n=5 in duplicate.

#### **3.4.4.3. Conversion Of Steroids And 7-Oxysterols By 11 $\beta$ HSD1 In Murine Aortic Rings**

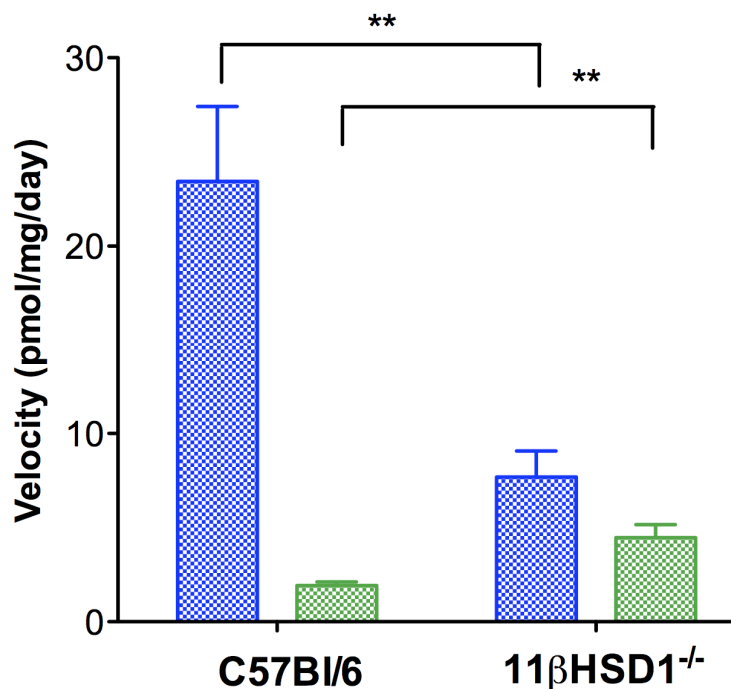
The pattern of metabolism of steroids by 11 $\beta$ HSD1 in murine aortic rings in culture was consistent with the nature of the reactions described for hepatic microsomes (see section 3.4.4.1). Reduction of 11-dehydrocorticosterone proceeded (on average 2.5x) faster than the dehydrogenation of corticosterone. Approximately 10 - 20% conversion was observed in the dehydrogenation direction after 24 h (Figure 3.4 – 12).

As expected, genetic disruption of 11 $\beta$ HSD1 resulted in a significantly lower rate of reduction of 11-dehydrocorticosterone but not complete ablation (see *footnote \**) Detection of product generated by dehydrogenation was expected due to the presence of 11 $\beta$ HSD2 in the vascular wall (Christy *et al.*, 2003) and unexpectedly, the velocity of 11 $\beta$ HSD2 reaction increased following the genetic disruption of 11 $\beta$ HSD1 as in Figure 3.4 – 12.

Formation of products of both reduction of 7-KC and dehydrogenation of 7 $\beta$ OHC was detected as shown in Figure 3.4 – 13. Interestingly, genetic disruption of 11 $\beta$ HSD1 resulted in a substantially significant reduction in the rate of metabolism of 7-oxysterols in both directions. Oxysterols were not metabolised by 11 $\beta$ HSD2 in the aortae. Lastly, 7-KC was not formed by aortic rings from either WT or KO mice incubated with medium containing 7 $\alpha$ OHC. Nor was 7 $\alpha$ OHC formed upon incubation of aortic rings with 7-KC.

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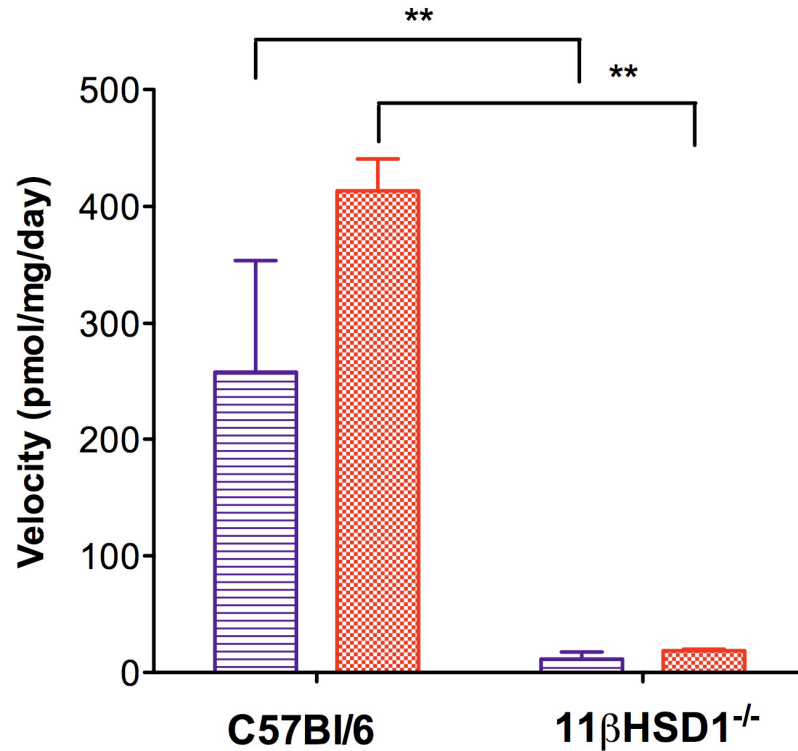
**NOTE:** \* Originally created 11 $\beta$ HSD1<sup>-/-</sup> mice (Kotelevtsev *et al.*, 1997) had targeted deletion of this gene (*HSD11b1*) for exons 3 and 4 (and thus expression) from P1 and P2 promoters. These promoters are widely expressed in most tissues of MF1 background mice. Once mice were backcrossed onto C57Bl/6 background of mice it appeared that some of the residual expression of 11 $\beta$ HSD1 protein is still observed, coming from a P1-promoter in tissues like lungs (~10-30%). Residual activity (~10%) of 11 $\beta$ HSD1 has later on also been observed in the vascular tissues from 11 $\beta$ HSD1<sup>-/-</sup> mice (Dover *et al.*, 2006)), where some of the 11 $\beta$ HSD1 protein made from this promoter may still be present. It is thus assumed that the residual activity comes from the P1 and P2 promoters if these promoters are used in vasculature, but these findings are currently under investigation in the laboratory.



**Figure 3.4-12 Metabolism of steroids by aortic rings from C57Bl/6 and 11βHSD1<sup>-/-</sup> mice.**

Metabolism of steroids by reduction (blue, 11-dehydrocorticosteron (A) → corticosterone (B)) and dehydrogenation (green, B→A) of substrates by 11βHSD isozymes by aortic rings from C57Bl/6 and 11βHSD1<sup>-/-</sup> mice was assessed following an overnight incubation (24h). Reduction of A was dominant in the C57Bl/6 mice and was significantly reduced in the 11βHSD1<sup>-/-</sup> mice. The dehydrogenation occurred at a faster rate in the 11βHSD1<sup>-/-</sup> mice compared to C57Bl/6. All data are mean ± SEM, compared using unpaired Student's t-test, n=10, \*\*p<0.01 vs. velocity of same reaction in tissue from C57Bl/6 mice.





**Figure 3.4-13 Conversion of 7-oxysterols by aortae from C57Bl/6 and 11βHSD1<sup>-/-</sup> mice.**

Metabolism of 7-oxysterols by reduction of 7-ketocholesterol (7-KC, purple) and dehydrogenation of 7β-hydroxycholesterol (7βOHC, red) by 11βHSD1 by aortic rings from C57Bl/6 and 11βHSD1<sup>-/-</sup> mice was assessed following an overnight incubation (24 h). Reduction and dehydrogenation occurred at a similar rate in aortic rings from C57Bl/6 mice but these velocities were both significantly reduced in the aortic rings from 11βHSD1<sup>-/-</sup> mice. However, residual activity was still detected in the 11βHSD1<sup>-/-</sup> vessels (values significantly different from zero). All data are mean ± SEM, compared using unpaired Student's t-test, n=6, \*\*p<0.01 vs. velocity of the same reaction in tissues from C57Bl/6 mice.

### 3.4.5. Does 11 $\beta$ HSD2 Inter-Convert 7-Oxysterols In Stably Transfected Cells?

This question was addressed first in utilising CHO cells stably transfected with human *HSD11B2* to generate h11 $\beta$ HSD2 recombinant protein. Dehydrogenation and reduction of steroids were tested using dexamethasone (DEX) and 11-dehydrodexamethasone (11-DHDEX) as substrates. These synthetic steroids were chosen since 11-DHDEX is a substrate for reduction by 11 $\beta$ HSD2, whereas 11-dehydrocorticosterone is not (Best *et al.*, 1997). The h11 $\beta$ HSD2 transfected in cells preferentially metabolised DEX to 11- DHDEX, while the reverse reaction catalysed by the same enzyme was recorded at very low rate, 40% vs. ~10 % conversion.

In the assay of 7-oxysterol conversion by recombinant h11 $\beta$ HSD2, a product was not generated from 7-KC (reduction), 7 $\alpha$ OHC or 7 $\beta$ OHC (both dehydrogenation), as seen in Table 3 - 7. Negative controls (non-transfected cells) were also included, in which conversion of neither steroids nor oxysterols could be detected.

To ensure the oxysterols were able to penetrate the CHO cells, both media and cells were analysed to ascertain the distribution of oxysterols post-incubation. The data were expressed as percentage product formed and percentage substrate remaining (Table 3 - 7). Overall around 80% of 7-oxysterols was recovered from the assay and distribution per cell or medium is expressed as a percentage of the total 7-oxysterols recovered. Most of 7-KC was recovered from media ( $97.1 \pm 0.03$  %) and very little penetrated the cells (< 2%). Of the total 7 $\beta$ OHC or 7 $\alpha$ OHC recovered, on average >60% was present in the cells and ~30% in the medium. Therefore, lipophilic oxysterols 7 $\alpha$ OHC and 7 $\beta$ OHC (not 7-KC) readily traversed the membrane of CHO cells, but did not bind to the 11 $\beta$ HSD2 enzyme, as seen by the lack of relevant product in the assay.

**Table 3-7 Lack of Metabolism of 7-Oxysterols by 11 $\beta$ HSD2**

Reaction	% Product	% Remaining substrate
7 $\alpha$ OHC $\rightarrow$ 7-KC	0	98.0 $\pm$ 0.7
7 $\beta$ OHC $\rightarrow$ 7-KC	0	96.4 $\pm$ 0.3
7-KC $\rightarrow$ 7 $\beta$ OHC	0	97.1 $\pm$ 0.1

This table demonstrates the lack of product formed by h11 $\beta$ HSD2 expressed in stably transfected CHO cells. Cells were treated with 7-oxysterols (1 $\mu$ M, 16 h) and the amount of product formed both in the cells and in the medium were measured and detected by GC/MS. The vast majority of each substrate was recovered and had not been converted to the anticipated product. All data are mean  $\pm$  SEM, n=4, in duplicate. 7 $\beta$ OHC, 7 $\beta$ -hydroxycholesterol; 7 $\alpha$ OHC, 7 $\alpha$ -hydroxycholesterol; 7-KC, 7-ketocholesterol.

#### 3.4.6. Does Renal 11 $\beta$ HSD2 Have A Role In Oxysterol Metabolism?

A second model was tested to assess whether oxysterols were substrates for murine 11 $\beta$ HSD2; the use of tissue homogenates avoided difficulties in access invoked by intact outer cell membranes. Murine kidneys contain both isozymes of 11 $\beta$ HSD and, therefore, reactions were performed using renal homogenates from 11 $\beta$ HSD1<sup>-/-</sup> mice.

Metabolism of steroids could be detected, confirming the integrity of the enzymatic preparation. Renal 11 $\beta$ HSD2 preferentially metabolised dexamethasone to 11-dehydrodexamethasone. The assay conditions of dehydrogenation and reduction of reactions (DEX  $\rightarrow$  11-DHDEX and 11-DHDEX  $\rightarrow$  DEX, respectively) were such to achieve 20-30% product formation (reaction velocities were not calculated).

In the assay of dehydrogenation of 7 $\beta$ OHC or 7 $\alpha$ OHC, a product was not formed (Table 3 - 8). Likewise, in the assay to test reduction of 7-KC, a product was not detected using HPLC although a very small percentage of 7 $\beta$ OHC was quantified (1.8  $\pm$  0.6%) using GC/MS, a technique with superior sensitivity. These data showed that 11 $\beta$ HSD2 does not play a role in the metabolism of 7-oxysterols to any

significant degree. Some depletion of 7 $\alpha$ OHC and 7 $\beta$ OHC was noticed, suggesting formation of an alternative product (although the presence of this product was not detected under the chromatographic conditions used), whereas substrates were fully recovered from blank samples containing buffer alone confirming chemical stability during the incubation.

**Table 3-8 Lack of metabolism of 7-oxysterols by renal 11 $\beta$ HSD2**

<b>Reaction</b>	<b>% Product</b>	<b>% Remaining substrate</b>
<b>7<math>\alpha</math>OHC <math>\rightarrow</math> 7-KC</b>	0	68.0 $\pm$ 2.8
<b>7<math>\beta</math>OHC <math>\rightarrow</math> 7-KC</b>	2.6 $\pm$ 2.4	56.2 $\pm$ 1.7
<b>7-KC <math>\rightarrow</math> 7<math>\beta</math>OHC</b>	1.8 $\pm$ 0.6	84.3 $\pm$ 3.2

This table demonstrates the lack of proposed product formed by m11 $\beta$ HSD2 in renal homogenates from 11 $\beta$ HSD1<sup>-/-</sup> mice. Very small amounts of proposed products were formed during incubation with 7 $\beta$ -hydroxycholesterol (7 $\beta$ OHC) and 7-ketocholesterol (7-KC). An unknown product was also formed in each assay, accounting for loss of substrate. All data are mean  $\pm$  SEM, n=4, in duplicate. 7 $\alpha$ OHC, 7 $\alpha$ -hydroxycholesterol.

### 3.5. Discussion

The work presented in this chapter tested the hypothesis that m11 $\beta$ HSD1 will accept 7-oxysterols as substrates. The initial aim of this work was to investigate the ability of 7-oxysterols to bind to and interact with the active site of m11 $\beta$ HSD1. *In silico* modelling suggested that the relevant functional groups assumed positions within the enzymatic active site to permit catalysis. Secondly, this work investigated the directionality of the 11 $\beta$ HSD1 enzyme utilizing 7-oxysterol as substrates in three different systems: recombinant protein, membrane associated enzyme from hepatic microsomes and an *ex vivo* preparation of murine aortae. Data obtained using all three systems demonstrate that murine 11 $\beta$ HSD1 can catalyze dehydrogenation of 7 $\beta$ OHC and 7-KC, whereas not all models allowed reduction to proceed. The directionality of individual reactions could also be influenced by cofactor supply. The direct comparison of this metabolism using tissues from C57Bl/6 and 11 $\beta$ HSD1<sup>-/-</sup> mice, demonstrated specificity of m11 $\beta$ HSD1 as being the only enzyme in the liver capable of executing these reactions with 7-oxysterols, as this reaction was abolished upon deletion of 11 $\beta$ HSD1. Lastly the kinetic data also highlight the differences between the characteristics of metabolism of 7-oxysterols and glucocorticoids by 11 $\beta$ HSD1: these interactions are further explored in Chapter 4.

The kinetics of metabolism of 7-oxysterols by m11 $\beta$ HSD1 had not been studied at the outset of the work leading to this thesis. Furthermore the kinetics of oxidation and reduction of 7-oxysterols had not been compared with those of glucocorticoids. In fact, it is only recently that the crystalised murine protein has become available (Zhang *et al.*, 2005) for *in silico* modelling of interactions of substrates and inhibitors with the active site. This advance has been driven through increased interest in 11 $\beta$ HSD1 as a therapeutic target for obesity and cardiovascular diseases, and various models of crystalized 11 $\beta$ HSD1 have now been generated for modelling purposes (Hosfield *et al.*, 2005).

### 3.5.1. *In Silico* Modeling Of 7-Oxysterol Binding To Murine And Human 11 $\beta$ HSD1

Comparison of murine and human enzymatic structures showed that a considerable similarity existed in the orientation of key active site residues (the catalytic tetrad). Indeed the catalytic sites of human, rat and murine 11 $\beta$ HSD1 share 79% homology with respect to amino acids and previously the least variation in the architecture of the active site (of those species tested) has been shown between human and murine 11 $\beta$ HSD1 (Hult, 2004). The cofactor binding sites are also virtually identical between the proteins from these species (Hult *et al.*, 2006). For these reasons, the murine enzyme is considered a good model for comparison of enzyme structure and substrate interaction *in silico*, closely mimicking the human enzyme and facilitating translational research. Furthermore the availability of transgenic murine models lacking 11 $\beta$ HSD1 merits further investigations of novel metabolism of this enzyme for potential therapy.

The catalytic tetrad of murine and human enzymes comprise Asn, Tyr<sup>183</sup>, Ser<sup>170</sup> and Lys residues and they require a cofactor (NADP<sup>+</sup>/NADPH) for catalysis. These highly conserved residues are orientated within the lipophilic substrate-binding pocket of the protein (Jörnvall *et al.*, 1995). This pocket is formed by the substrate-binding loop, which resembles a tunnel through which substrates gain access to the active site. It was previously hypothesized that this enzyme's location within microsomal membranes favours binding of lipophilic substrates (such as 7 $\beta$ OHC and cortisol) through the substrate-binding pocket (Ogg *et al.*, 2005).

A close model to m11 $\beta$ HSD1 (m7 $\alpha$ HSD bound to a 7-oxysterol-like molecule, PDB ID: 1FMC) represented a great resource with which to simulate potential steric positioning of the oxygenated groups of 7-oxysterols and their orientation in the binding tunnel of 11 $\beta$ HSD1. This was simply an approach to approximate the putative binding of 7-oxysterols to the active site of 11 $\beta$ HSD1, since the crystal structure of m11 $\beta$ HSD1 combined with the 7-oxysterols was not available at the time (and remains unavailable). More refined positioning of the substrates into the active

site of PDB model of m11 $\beta$ HSD1 was performed by generating the energy maps for ligand-protein interaction. These have allowed exact positioning of substrate within suggested site points in the binding pocket of a protein. By further performing 3-D docking of substrates into the active site of m11 $\beta$ HSD1 (as described in section 3.4.1) the distances between key active residues and side chains were obtained. It became apparent that 7 $\beta$ OHC and 7-KC, but not 7 $\alpha$ OHC, may bind to m11 $\beta$ HSD1 with sufficient proximity to permit catalysis, as the interacting residues were within acceptable hydrogen bond distance. The differences between molecular structures of 7 $\beta$ OHC and 7-KC were examined using 3-D reconstructions. The orientation of both 7-KC and 7 $\beta$ OHC suggested favourable binding to the active site of 11 $\beta$ HSD1, although chair orientation of 7 $\beta$ OHC resulted in its 7 $\beta$ -hydroxy group being located slightly further from Tyr<sup>183</sup> residue in the active site in the mouse than the 7-ketone of 7-KC.

To further explore the *in silico* predictions of the suitability of these 7-oxysterols as substrates for 11 $\beta$ HSD1, kinetics were investigated using a series of complementary *in vitro* and *ex vivo* model systems.

### 3.5.2. *In Vitro* And *Ex Vivo* Assessment Of 11 $\beta$ HSD1 Activity

Having established that both 7 $\beta$ OHC and 7-KC were likely ligands for m11 $\beta$ HSD1, their rates of inter-conversion were tested *in vitro* and *ex vivo*. All models of m11 $\beta$ HSD1 were able to accept 7 $\beta$ OHC, but not 7 $\alpha$ OHC, as a substrate, and convert it to 7-KC; thus confirming the prediction provided by the *in silico* analysis. At the outset it was not certain whether reduction of 7-KC by m11 $\beta$ HSD1 would generate more than one product (7 $\alpha$ OHC and/or 7 $\beta$ OHC). In this work stereo-specificity for the formation of the 7 $\beta$ -isomer over 7 $\alpha$ OHC was established. The data presented show that most (but not all) models used were able to reduce 7-KC to 7 $\beta$ OHC to some degree but 7 $\alpha$ OHC was not formed under any circumstances tested. Thus, the substrate specificity observed here indicates that m11 $\beta$ HSD1 is similar to the human and rat enzyme in substrate preference, as described in the literature (Hult, 2004). Only 11 $\beta$ HSD1 in hepatic microsomes from the hamster does not exert this stereo-specificity and forms both 7 $\alpha$ OHC and 7 $\beta$ OHC upon reduction of 7-KC (Maeda *et al.*, 2006). Sequence alignment, as performed and presented in Figure 3.4 – 2

highlights some of the differences between enzyme from three different species, but the catalytic parts of the sequences share very high homology. Further insight to this difference might be obtained by comparison of tertiary structure of the hamster protein with those of human and mouse structures.

The rates of metabolism of 7-oxysterols by m11 $\beta$ HSD1 were compared with those of glucocorticoids. As expected and by design, m11 $\beta$ HSD1 metabolised glucocorticoids in all *ex vivo* and *in vitro* model systems used and reduction predominated, consistent with the literature (Kotelevtsev *et al.*, 1997; Lavery *et al.*, 2006). Interestingly, reduction of 7-KC by m11 $\beta$ HSD1 was detected in some test systems but not others, whereas dehydrogenation of 7 $\beta$ OHC could be measured consistently.

Mirroring the pattern for glucocorticoids, murine recombinant 11 $\beta$ HSD1 inter-converted 7-oxysterol as substrates in both directions but with a preference for reduction of 7-KC. However, while this approach proved very useful for direct comparison of the ability of 11 $\beta$ HSD1 to use oxysterols and steroids as substrates, it reflected a scenario distinct from the natural physiological environment where 11 $\beta$ HSD1 is membrane bound. To address this issue, further reactions were carried out using HEK293 cells stably transfected to generate m11 $\beta$ HSD1 and primary tissues *ex vivo* naturally expressing 11 $\beta$ HSD1; thus moving the focus from the purified protein to the enzyme specifically associated with membranes.

In these further model systems, 7-oxysterols were still metabolised less efficiently by 11 $\beta$ HSD1 compared with glucocorticoids, characterised by slower rates of reaction and much higher binding constants ( $K_m$  in high  $\mu$ M vs. low  $\mu$ M, respectively). These findings, however, do not necessarily mean that 11 $\beta$ HSD1 will have a lesser role to play in oxysterol over glucocorticoid metabolism, as the velocities of enzymatic reactions are determined in part by substrate concentrations. In atherosclerosis the levels of oxysterols exceed those of glucocorticoids by three orders of magnitude (from low nM (GC) to low  $\mu$ M (oxysterols)) (Brown and Jessup, 2009) and thus there may be significant turnover of 7-oxysterols.



In systems utilising membrane bound 11 $\beta$ HSD1, differences between metabolism of the two types of substrate were revealed with respect to the equilibrium set-point between reduction and dehydrogenation. It is well established that, within a suitable cellular environment, reduction is the preferred direction of 11 $\beta$ HSD1 for metabolism of glucocorticoids (Atanasov *et al.*, 2004; Bujalska *et al.*, 2005; Lavery *et al.*, 2006).

However, the data presented here show that lower  $K_m$  values were consistently obtained for dehydrogenation of 7 $\beta$ OHC compared with those for reduction of 7-KC, implying the enzyme's preference to act as a dehydrogenase with this class of substrates. This phenomenon was even more pronounced in hepatic microsomes in which reduction of 7-KC by 11 $\beta$ HSD1 could not be invoked. This is surprising given that in this model 11 $\beta$ HSD1 is orientated within the microsomal lumen in close proximity to H6PDH (Balázs *et al.*, 2009; Bujalska *et al.*, 2005), which is normally sufficient to provide excess NADPH co-factor to drive reduction, when supplied with G6P and NADP<sup>+</sup>. Certainly, *in vitro* studies by Frick *et al.*, (2004) using wild-type recombinant 11 $\beta$ HSD1 in intact cells, engineered to face into the lumen of ER, and a mutant K5S/K6S 11 $\beta$ HSD1 engineered to face the cytoplasm, demonstrated that the 11 $\beta$ HSD1-luminal orientation was essential for efficient reduction of 7-KC.

A number of approaches were taken to ensure the integrity of the microsomal preparations in order to establish the right conditions for 7-KC reduction to occur. In each assay, conditions were used (e.g. high concentration of NADPH, use of alamethicine) which allowed reduction of glucocorticoids to proceed efficiently and predominate over dehydrogenation, indicating appropriate handling of the tissue. In the face of compelling evidence, still only a very small percentage of 7-KC was reduced (as detected using GC/MS, seen in Table 3 – 5) by 11 $\beta$ HSD1 in hepatic microsomes. For this purpose, a cofactor generating system was added and alternative co-factors (NADH in place of NADPH) were explored. These manipulations did not succeed in promoting reduction of 7-KC. It was possible that higher concentrations of NADPH were required for reduction of 7-KC in comparison to those used in experiments involving reduction of glucocorticoids. On the other

hand both  $\text{NADP}^+$  and  $\text{NAD}^+$  cofactors were also accepted by m11 $\beta$ HSD1 in the formation of 7-KC from 7 $\beta$ OHC, as is the case for conversion of glucocorticoids (Christy, 2003). Indeed, in many species  $\text{NADP}^+/\text{NADPH}$  as opposed to  $\text{NAD}/\text{NADH}$  is the preferred set of cofactors for 11 $\beta$ HSD1, but in the mouse there is less clear distinction between these two cofactors for the glucocorticoid metabolism (Arnold *et al.*, 2003).

To further address the concern over lack of reducing cofactor *in vitro*, HEK293 cells were co-transfected with human 11 $\beta$ HSD1 and H6PDH enzymes (collaboration with (Dakin, 2008)). Although in control cells the proportions of reduction and dehydrogenation reactions were relatively closely matched (60 : 40% respectively), upon co-expression (Figure 3.5 – 1), glucocorticoid metabolism proceeded mainly as a reductase and dehydrogenation was significantly reduced (80 : 20 % respectively). A significant increase in 7-KC reduction was also observed under the same co-expression conditions and dehydrogenation of 7 $\beta$ OHC was suppressed, but it nevertheless remained the dominant reaction over reduction of 7-KC (Dakin, 2008). Overall these findings suggest that metabolism of 7-oxysterols by 11 $\beta$ HSD1 is less dependent on H6PDH in determining its kinetics as compared to glucocorticoids, and other factors must be at play to allow dehydrogenase activity of 7 $\beta$ OHC to 7-KC to persist (Maeda *et al.*, 2006). It might be informative to repeat some of these experiments in primary hepatocytes, which have high expression of H6PDH. One of the concerns with these cells as a model, however, is the existence of highly active competing pathways: various aldo- and keto- reductases and CypP450s in hepatocytes which may also contribute to metabolism of oxysterols (Jin and Pennings, 2007).

A further factor that could have impaired reduction is a barrier to access of 7-KC into the cell and microsomal lumen. During the inter-conversion assay using HEK293 cells, it became apparent that oxysterols were sequestered in the cells. They could be extracted from both media and the cells, whereas most steroids (> 95 %) were recovered from the medium only. However 7 $\beta$ OHC was rapidly sequestered whereas 7-KC was mainly found in the medium. Thus the substrate for reduction may have

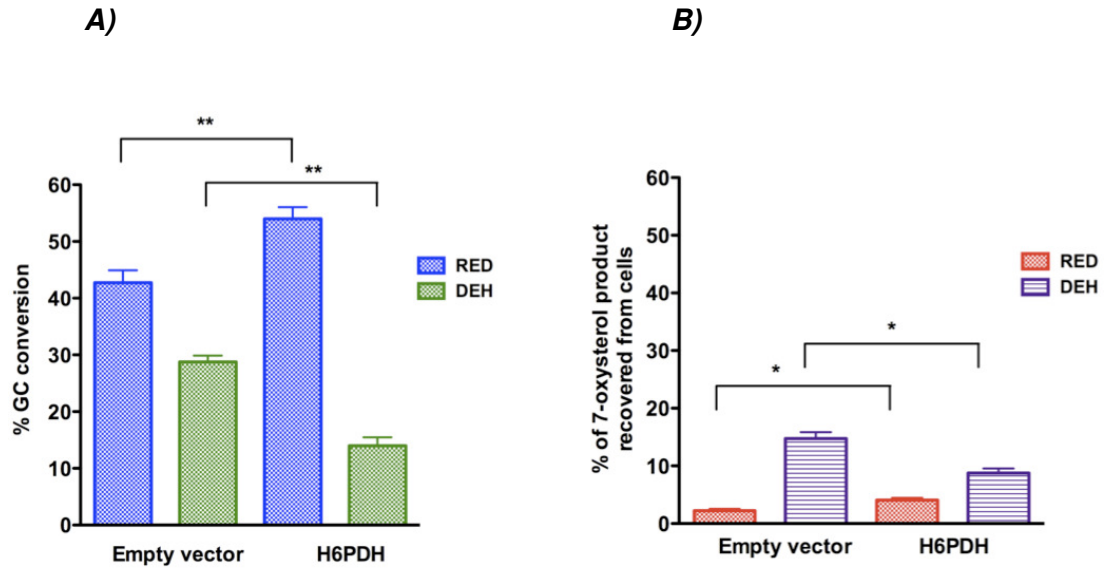
been prevented from entering the cell in substantial quantities. Indeed

7-KC generated by dehydrogenation of 7 $\beta$ OHC appeared also to be rapidly exported back into the medium. Little is known about transport of 7-oxysterols across outer cell membranes (Olkkonen *et al.*, 2006) or into the microsomal compartment, although active transporters exist in the liver, which facilitate the movement of other oxysterols and cholesterol (Baldan *et al.*, 2009; Gelissen *et al.*, 2006) in a highly regulated manner.

HEK293 cells, which are derived from kidney (Graham *et al.*, 1977) may not express the correct transporters to allow efficient entry of 7-KC and perhaps the experiments may be repeated in a hepatic cell line. One would, however, predict that their lipophilic nature would allow them to passively penetrate the cell membrane.

To explore penetration of the microsomal membrane further, a membrane permeabilising agent (allameticine) (Banhegyi *et al.*, 2004) was included in the assays to facilitate 7-KC transport across the ER membrane. Furthermore, it was postulated that a longer incubation was required to allow the potentially slower (compared with glucocorticoids) diffusion of oxysterols through the microsomal membrane. However, even when this was attempted, reduction was still not observed. Concerns were raised regarding heat-induced degradation of sterols during permeabilisation of microsomes for periods longer than 2h (reviewed by Schroepfer, (2000)) and thus longer incubations were avoided.

The transport of 7-KC across the microsomal membrane may be dependent on various associated proteins (such as Sterol Regulatory Element Binding Proteins (SREBPs), to which 7-KC can bind *in vivo* (Brown *et al.*, 2002). These may not have been present or active in the microsomal preparations, although in cells SREBP transcription factors are usually associated with the lumen of endoplasmic reticulum or Golgi apparatus in their unstimulated state (Espenshade *et al.*, 1999).



**Figure 3.5-1 Metabolism of steroids and oxysterols by 11 $\beta$ HSD1 in stably transfected HEK293 cells.**

HEK293 cells stably expressing the gene for h11 $\beta$ HSD1 were transiently transfected with human hexose 6-phosphate dehydrogenase (hH6PDH). Data are expressed as a % of conversion of substrate (200 nM steroid, 1  $\mu$ M oxysterols). **A)** Velocities of reduction (RED) of 11-dehydrocorticosterone to corticosterone, (blue) and dehydrogenation (DEH) of corticosterone (green) were measured. In control cells the proportions of reduction and dehydrogenation reactions were similar (60 : 40%, respectively) but upon co-expression, glucocorticoid metabolism proceeded mainly as a reductase (80 : 20%, respectively). **B)** Metabolism of 7-oxysterols by reduction (RED) of 7-ketocholesterol (red) and dehydrogenation (DEH) of 7 $\beta$ -hydroxycholesterol (7 $\beta$ OHC, purple) by h11 $\beta$ HSD1 proceeded in favor of dehydrogenation of 7 $\beta$ OHC. Upon co-transfection with 11 $\beta$ HSD1 and H6PDH a significant increase in reduction of 7-KC and decrease in dehydrogenation of 7 $\beta$ OHC were observed compared with control, however dehydrogenation of 7 $\beta$ OHC was still the dominant reaction. All data are mean  $\pm$  SEM, compared using unpaired Student's t-test, n=6, in duplicate, \*\*p<0.01, \*p<0.05. GC, glucocorticoid.

Certainly the transcriptional regulation of these processes would not have been active in tissue homogenates. HEK293 cells express all three isoforms of SREBP: SREBP1a, SREBP1c and SREBP2 (Hannah *et al.*, 2001), although they are subject to feedback regulation by cholesterol, which blocks the proteolytic release of SREBP molecules from the membrane. Sterols have their greatest inhibitory effects on SREBP-2. It would appear that HEK293 cells may be able to carry out the transport of 7-KC, yet there was still a lack of reduction of 7-KC by 11 $\beta$ HSD1.

There are reports showing that 7-KC interferes with the structured phospholipid bilayer of the cell membranes and perhaps of the ER membrane (Massey and Pownall, 2006), causing significant changes in the biophysical properties of the lipid bilayer, by locating close to the membrane interface. 7-KC may embed itself between the non-polar lipid tails in the ER membrane where it has a tendency to form crystals (Massey, 2006), and as a result, the transport of 7-KC may have been impaired across the membrane into the lumen of ER. Crystal formation may also have disrupted the association between 11 $\beta$ HSD1 and H6PDH, preventing the coupling necessary to allow reduction to proceed. In Chapter 4 however, data will be presented showing that reduction of glucocorticoid can still take place efficiently in the presence of high concentrations of 7-KC so this is unlikely to be the explanation. It still remains unclear why only glucocorticoid metabolism has occurred under the same conditions. The balance to favour reduced and oxidized cofactor may simply be achieved better *in vivo* (Braakman *et al.*, 1992), because of constant provision of cofactor by the regulatory enzymes sensing the redox state of the ER and responding to demand.

One of the questions raised in this Chapter was whether 11 $\beta$ HSD1 was the only enzyme capable of catalysing the inter-conversion of 7 $\beta$ OHC and 7-KC. To address this, the use of 11 $\beta$ HSD1<sup>-/-</sup> animals was vital. Since formation of reaction product was not detected in the microsomes from 11 $\beta$ HSD1<sup>-/-</sup> mice, this suggests that 11 $\beta$ HSD1 is the only enzyme metabolizing 7-oxysterols in this compartment of hepatocytes. Further, there was not an obvious involvement of 11 $\beta$ HSD2 in metabolizing 7-oxysterols since renal homogenates of 11 $\beta$ HSD1<sup>-/-</sup> mice were unable

to inter-convert 7-oxysterols. Previously, and also reported here, human HEK293 cells transfected with 11 $\beta$ HSD2 could not convert 7-KC (Schweizer *et al.*, 2004). To an extent, these findings are in line with those of carbenoxolone inhibiting 7-oxysterol metabolism, observed by Schweizer *et al.*, (2004), although carbenoxolone is not a selective inhibitor as it inhibits both isozymes of 11 $\beta$ HSD, and also affects prostaglandin synthesis (Walker and Andrew, 2006). The above data showing dual directionality of 11 $\beta$ HSD1 suggest that the dehydrogenase activity of this enzyme may contribute to accumulation of 7-KC in for example macrophages and vascular cells. The *in vitro* findings should of course be carefully extrapolated onto an *in vivo* system, where many additional factors influence reaction direction, enzyme expression and enzyme structure. It has been speculated that inhibition of 11 $\beta$ HSD1 may reverse the lysosomal accumulation of 7-KC (Brown *et al.*, 2000b; Jessup and Brown, 2005), supporting the data in this chapter that 11 $\beta$ HSD1 predominantly performs dehydrogenation of 7 $\beta$ OHC to 7-KC. The dehydrogenation reaction of 11 $\beta$ HSD1 may thus contribute to 7-KC accumulation, leading to its further deleterious effects on tissue.

Findings from the work in this chapter support previous assertions in the literature that 11 $\beta$ HSD1 is providing a major route of oxysterol metabolism in the liver (Jessup and Brown, 2005), and show that indeed this may be the case in other tissues too, including the vasculature. Previously investigations of 11 $\beta$ HSD1 in aortic rings have only utilised glucocorticoids as substrates (Dover *et al.*, 2006; Morris *et al.*, 2003); thus aortic rings from 11 $\beta$ HSD2<sup>-/-</sup> mice, as a source of 11 $\beta$ HSD1, were used to determine their ability to metabolize either 7 $\beta$ OHC or 7-KC. The virtual absence of product following incubation of oxysterols with vessels from 11 $\beta$ HSD1<sup>-/-</sup> mice confirmed *in vitro* findings by others (Schweizer *et al.*, 2004) that 11 $\beta$ HSD2 does not metabolise 7-oxysterols. Hence within the vessel inter-conversion of oxysterols will be catalysed solely by 11 $\beta$ HSD1, which will dictate the balance of these species and potentially regulate and differentiate the biological functions or toxic actions they possess.

There are several implications from the data presented in this chapter. Firstly, the dominance of reduction over dehydrogenation is less marked for 7-oxysterols than for glucocorticoids, and indeed, under some circumstances, dehydrogenation occurs exclusively. If 7-KC and 7 $\beta$ OHC have differential physiological effects, then these observations are important to understand the role of 11 $\beta$ HSD1 in determining the balance of their local levels and actions. It will be important to extrapolate these findings to an *in vivo* model. Since 11 $\beta$ HSD1 may metabolize both glucocorticoid and 7-oxysterols, competition may occur between these pathways *in vivo* depending on the local concentrations of each substrate. This work is further explored in Chapter 4. Lastly, it is important to establish whether 7-oxysterols have distinctive biological effects, which may be regulated via inter-conversion by 11 $\beta$ HSD1.

## **Chapter 4. Interaction Between Metabolism Of 7-Oxysterols And Glucocorticoids By 11 $\beta$ HSD1**



## 4.1. Introduction

The findings from the previous chapter established that 7-oxysterols are substrates for m11 $\beta$ HSD1. However, whilst 11 $\beta$ HSD1 inter-converts 7-oxysterols, the preferred reaction direction is different from that of glucocorticoids. Since 7-oxysterols bind to 11 $\beta$ HSD1, they may compete with glucocorticoids for metabolism by the this enzyme (Balázs *et al.*, 2009; Robinson *et al.*, 2003). Consequently, through substrate competition for 11 $\beta$ HSD1, 7-ketocholesterol (7-KC) or 7 $\beta$ -hydroxycholesterol (7 $\beta$ OHC) may modulate glucocorticoid production by 11 $\beta$ HSD1 and, thereby, its activity in target tissues. Since pre-receptor metabolism of glucocorticoids by 11 $\beta$ HSD1 regulates access of glucocorticoids to the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) (Tomlinson *et al.*, 2004a), the modulation of glucocorticoid metabolism by 7-oxysterols may also influence activation of these receptors. The directionality of 11 $\beta$ HSD1 may also be regulated by these small lipids (in addition to the well-known regulation of directionality conferred by H6PDH (Atanasov *et al.*, 2004)) through competition for cofactor. Such regulation maybe of particular importance in atherosclerosis, for example, which is associated with increased levels of oxysterols which are readily sequestered into macrophages and perivascular adipocytes (Kritharides, 1995) both of which express 11 $\beta$ HSD1.

The nature of the competition between these alternative and conventional substrates for 11 $\beta$ HSD1 has not been studied to date. However there is limited evidence of these types of interactions from studies of other 7-oxygenated substrates; it has been shown that 7-neurosteroids can act as inhibitors of human 11 $\beta$ HSD1 (Nashev *et al.*, 2007); although this report does not fully elucidate the nature or the magnitude of this interaction. Recent work with other 7-hydroxylated compounds related to steroids also report competition between different substrates for metabolism by 11 $\beta$ HSD1 (Robinson *et al.*, 2003). For example, bile acids such as chenodeoxycholic acid (CDCA) can inhibit 11 $\beta$ HSD1 activity (Escher *et al.*, 1998; McNeilly *et al.*, 2006). More recently, work performed during the execution of this thesis, showed that 7-KC was more potent than 7 $\beta$ OHC at inhibiting the conversion of

glucocorticoids by 11 $\beta$ HSD1 in human adipocytes (Wamil *et al.*, 2008). However, in the same study the effect of 7-oxysterols on dehydrogenation of glucocorticoids by 11 $\beta$ HSD1 was not tested.

## 4.2. Research Hypothesis And Aims

The work in this chapter addressed the hypothesis that 7-oxysterols inhibit metabolism of glucocorticoids by 11 $\beta$ HSD1. Secondly, it tested the hypothesis that the preferred direction of glucocorticoid metabolism by 11 $\beta$ HSD1 will be influenced *in vitro* by the cellular levels of cholesterol, the precursor of 7-oxysterols.

The specific aims for the work described in this chapter were:

- To determine whether 7-oxysterols inhibit glucocorticoid metabolism by 11 $\beta$ HSD1;
- To establish the kinetic parameters to describe this competition;
- To determine whether an increase in, or reduction of, cellular levels of cholesterol and 7-oxysterols alters the kinetics of 11 $\beta$ HSD1-mediated glucocorticoid metabolism.

## 4.3. Methods

### 4.3.1. Inhibition Of 11 $\beta$ HSD1-mediated Reactions

The following model systems were used to investigate inhibitory kinetics:

- 1) Recombinant m11 $\beta$ HSD1 protein,
- 2) Recombinant m11 $\beta$ HSD1 protein generated in stably-transfected HEK293 cells,
- 3) Murine hepatic microsomes.

#### 4.3.1.1. **Preparation Of Oxysterol Solutions**

All oxysterols were prepared in ethanol containing butylated hydroxytoluene (BHT; 50 µg/ml), as described (section 2.2.2). The stock solution (30 mM, under argon) was further diluted in ethanol to achieve a 10 mM stock (Lizard *et al.*, 1999). Serial dilutions were prepared in phosphate buffer or in culture medium (DMEM).

For microsomal assays assessing the ability of 7-oxysterols to inhibit glucocorticoid conversion, solutions representing a range of concentrations of 7-oxysterols were prepared in ethanol, of which 5 µl was added to each assay, to obtain final concentrations of  $10^{-10}$  -  $10^{-4.5}$  M.

For tissue culture experiments, stock solutions of individual oxysterols (section 2.2.2) were prepared in DMEM. Of these stocks, an appropriate volume (2 ml/well) was added to the cells to obtain final concentrations of  $10^{-10}$  -  $10^{-4.5}$  M). A mixture of oxysterols containing 19-OHC, 22-OHC, 25-OHC, 27-OHC and 7-oxysterols was prepared as a stock solution, by combining aliquots of each (10 mM, 500 µl in ethanol) under argon, and vortexing (RT, 10 sec). The solvent was removed by evaporation (argon, RT) and dried residue was dissolved in ethanol (500 µl, containing 50 µg/ml BHT). The stock of mixture of oxysterols was sealed under argon, sonicated (on ice, 10 min) then vortexed (400 rpm, RT, 10 min) until fully dissolved. Further dilutions of oxysterols were made in DMEM or buffer, as appropriate.

#### 4.3.1.2. **Inhibition of Dehydrogenation of Corticosterone By 7-Oxysterols**

**a) Characterisation of inhibitory kinetics by Dixon Plot:** recombinant protein (20 µg/ml) was incubated with cofactor ( $\text{NADP}^+$ , 2 mM) as in section 2.5.2, and in the presence of four concentrations of substrate corticosterone (B; 5 µl; 20, 25, 100 and 200 nM) plus each of four different concentrations of inhibitor (7βOHC; 5 µl; 1, 2, 5 and 10 µM).

**b) Assessment of  $IC_{50}$  in intact HEK293 cells stably-transfected to produce m11 $\beta$ HSD1.** Cells were exposed to medium containing radioactive substrate ( $[^3H]_4$ -B, 30 nM) as in section 2.6.2. Assays were performed in 12-well plates ( $10^5$  cells/ml/well) and individual oxysterols (5  $\mu$ l/per well, final concentration  $10^{-10}$  –  $10^{-4.5}$  M, 0.01% ethanol control) were added to the medium together with the glucocorticoid (30 nM) and incubated; typically for 45 – 60 min. After incubation, cell morphology was checked by light microscopy, since supra-physiological concentrations of oxysterols had been used.

**c) Assessment of inhibition ex vivo:** was performed using murine hepatic microsomes (260  $\mu$ g/ml) as in section 2.7.1. The velocity of 11 $\beta$ HSD1 oxidation of B  $\rightarrow$  A, (B, 20 nM) was assessed in the presence of various concentrations of 7-oxysterols (5  $\mu$ l, 7-KC, or 7 $\beta$ OHC, final concentration  $10^{-10}$  –  $10^{-4.5}$  M) as an inhibitor. Upon obtaining concentration of 7 $\beta$ OHC that caused 50% inhibition ( $IC_{50}$ ) further kinetic parameters of metabolism of B (20 nM – 2  $\mu$ M) in the presence of single concentration of 7 $\beta$ OHC ( $IC_{50}$ ) or vehicle (0.01% ethanol control) and using protein concentration as above were also determined. Michaelis-Menten and Lineweaver-Burke Analysis were performed as described in section 4.3.1.

#### 4.3.1.3. **Inhibition Of Reduction Of 11-Dehydrocorticosterone By 7-Oxysterols**

**a) Characterisation of inhibitory kinetics by Dixon Plot:** recombinant m11 $\beta$ HSD1 (20  $\mu$ g/ml) was incubated (30 min, RT) with cofactor (NADPH, 2 mM) as in section 2.5.3 and in the presence of four concentrations of substrate, 11-dehydrocorticosterone (A) (500 nM, 1, 5, 10  $\mu$ M) with each of four concentrations of inhibitor (7-KC, 5  $\mu$ l of 500 nM, 1, 2, 10 and 20  $\mu$ M).

**b) Assessment of  $IC_{50}$  in HEK293 cells stably-transfected to produce m11 $\beta$ HSD1.** Cells were exposed to medium containing radioactive substrate ( $[^3H]_4$ -A, 30 nM) as described in section 2.6.4. Assays were performed on 12-well plates (1 ml/well) and individual oxysterols (5  $\mu$ l/per well, final concentration  $10^{-9}$ - $10^{-4.5}$  M, 0.01% ethanol

control) were added, together with unlabelled A (30 nM) and incubated; typically for 45 – 60 min.

**c) Assessment of inhibition in vitro:** was performed using murine hepatic microsomes (260 µg/ml) as in section 2.7.3. The velocity of the reduction of A → B (A, 20 nM) was measured in the presence of various concentrations (sections 4.3.1.2) of 7-oxysterols as inhibitors (5 µl,  $10^{-10}$  –  $10^{-4.5}$  M).

#### **4.3.2. Detection Of Oxysterols**

Detection of steroids and oxysterols was achieved using HPLC with UV- and on-line scintillation detection as described (sections 2.12.1 and 2.12.2, respectively).

#### **4.3.3. Controls And Quantitation**

All measurements of enzyme velocity in sections 4.3.1 and 4.3.2 were carried out within the linear range of substrate-to-product formation (<30% substrate conversion). Each experiment was carried out in duplicate and controls of vehicle (ethanol, 0.01%) alone were included.

#### **4.3.4. Cholesterol Manipulation *In Vitro***

Cellular cholesterol levels were manipulated by utilizing the cholesterol-carrier, methyl-β-cyclodextrin (MβCD). HEK293 cells, transfected with *HSD11B1* to generate m11βHSD1 recombinant protein (section 2.6.1), were plated on 6-well plates and incubated overnight in medium containing stripped serum (10%). The next day cells were maintained in serum-free media (DMEM, 1h), before being exposed to the following treatments:

##### **4.3.4.1. Cholesterol Depletion**

For cholesterol depletion, cells were treated with MβCD (10 mM, in DMEM, 37°C, 1h), as this was expected to reduce membrane cholesterol by ~89% (Francis and et al, 1999). According to previous work utilizing the same procedure (Danthi and Chow, 2004), cells maintained reduced cholesterol levels for at least 48 h following

30 – 60 min treatment. Since cellular cholesterol levels were successfully reduced after 1h incubation, this time point was used in further experiments. Control cells treated with DMEM only were also included on the same plate.

#### 4.3.4.2. **Cholesterol Loading**

To increase cholesterol levels in the cell membrane, M $\beta$ CD (5 mM, in DMEM, 37°C, 1h) was prepared in a complex with cholesterol (200  $\mu$ g/ml) to give Cholesterol:M $\beta$ CD at a molar ratio of 1:8, as described by Klein *et al.*, (1995). Cholesterol (10 mg/ml) was dissolved in chloroform:methanol (2:1, v/v, 1 ml), then transferred to a Falcon tube containing M $\beta$ CD (5 mM, 66.9 mg) and chloroform (19 ml), vortexed (RT, 1 min), sonicated (RT, 3 min) and solvent was evaporated under argon. The dried residue was re-solubilised in DMEM (20 ml) and incubated in a water bath (60 rpm, 37°C, 16 h) (Levitan *et al.*, 2000), in order to form a cholesterol complex chelated with M $\beta$ CD (chol-M $\beta$ CD, 1:8, 5mM). After incubation, the mixture was subject to double filtration (as in section 2.6.1.1) and the filtrate used for subsequent cell treatment. However, using this initial method of complex formation, the levels of cholesterol remained the same between control and treated cells ( $67 \pm 9$  vs.  $72 \pm 12$   $\mu$ g/mg protein, control vs. loading) and thus an alternative approach was sought.

A new method of augmentation of cellular cholesterol levels was applied by treatment with a commercially-available, cholesterol-loaded M $\beta$ CD (chol:M $\beta$ CD, 5 mM), termed “water-soluble cholesterol”, at a molar ratio 1:6 chol:M $\beta$ CD). “Water-soluble” cholesterol was dissolved in DMEM (0.01% ethanol) to obtain a 10 mM solution. Control cells were treated with serum-free DMEM. A further set of cells was subject to cholesterol loading followed by exposure to M $\beta$ CD (10 mM, 37 °C, 30 min and 1h) to subsequently reduce cellular cholesterol levels.

#### 4.3.4.3. **11 $\beta$ HSD1 Activity - Steroid Conversion**

Following either depletion of (section 4.3.3.1), or loading with (section 4.3.3.2) cholesterol, cells were washed in DMEM (37°C), then in PBS (4°C) and measurements of 11 $\beta$ HSD1 activity were performed. Cells ( $5 \times 10^6$  cells/well) were

incubated in serum-free DMEM containing [ $^3\text{H}$ ] $_4\text{-B}$  or [ $^3\text{H}$ ] $_4\text{-A}$  (30 nM per well, 1 h) for subsequent measurement of dehydrogenation or reduction respectively by 11 $\beta$ HSD1, as described (sections 2.6.2 and 2.6.4, respectively). Steroids were extracted from the media (section 2.6.2.1) and analysed as in section 2.12.2.1.

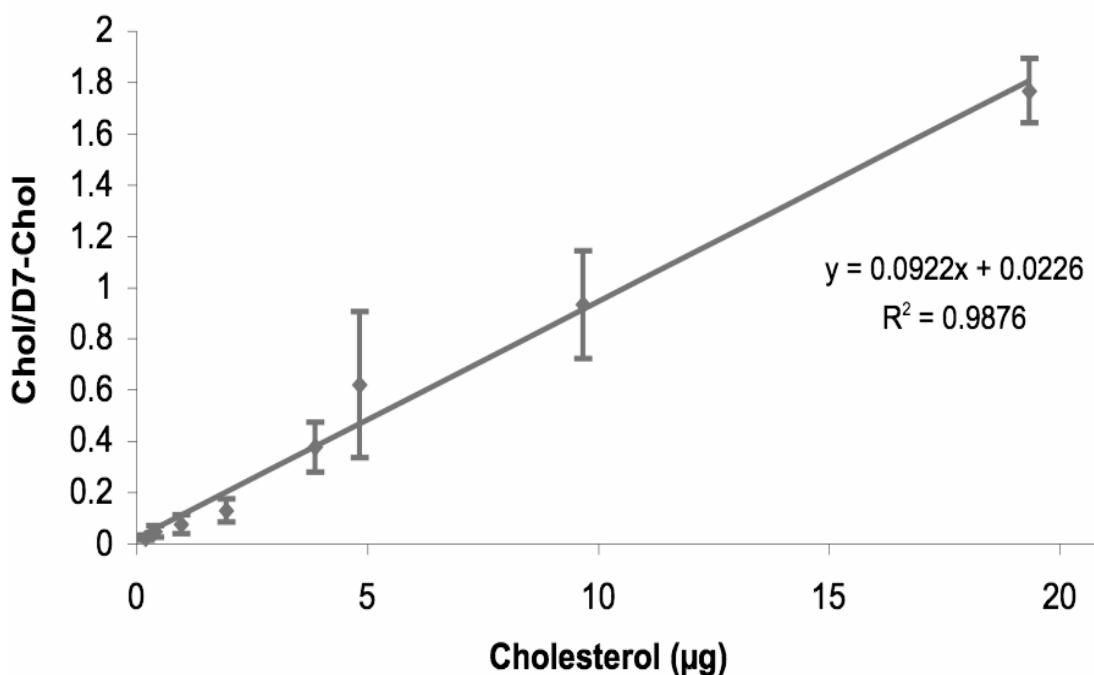
#### 4.3.4.4. **Determination Of Cholesterol And 7-Oxysterol Levels**

Cells were washed in PBS (4°C) then lysed (NaOH (200  $\mu\text{M}$ ), 0.6 ml/well) by rocking the plate (60 rpm, 4°C, 20 min) (Brown *et al.*, 1997), and an aliquot of cellular lysates (100  $\mu\text{l}$ ) was removed for determination of protein concentration (section 2.4.7). The remaining cellular lysate was used for extraction of cholesterol and 7-oxysterols (sections 4.3.3.2 and 2.6.3.2). The organic phases were evaporated (argon, RT) and samples stored at -20°C until analysis by GC/MS (section 2.13.2).

#### 4.3.4.5. **Controls And Quantitation**

For quantitation of oxysterols and cholesterol in cells depleted of, or loaded with, cholesterol, deuterium-labelled internal standards (d7-7-KC, d7-7 $\beta$ OHC, 50  $\mu\text{l}$ ) and d7-cholesterol (10  $\mu\text{g}$ /10  $\mu\text{l}$ ) were added to cells and media (sections 2.6.3.1 and 2.6.3.2).

For quantitation of cholesterol, a standard curve representing a range of concentrations of cholesterol (0.5, 1, 2.5, 5, 10, 12.5, 20, 50 and 100  $\mu\text{M}$ ) was prepared. Medium (1 ml) containing cholesterol (10  $\mu\text{l}$ ) and oxysterols (10  $\mu\text{l}$ ) in the absence of cells was incubated for the duration of treatment (section 4.3.4.3), on a 24-well plate, in duplicate and all other controls were included, as described (section 2.6.6). A new standard curve was generated during each assay to control for differences in incubation and extraction. An exemplar standard curve of cholesterol is shown (Figure 4.3 – 1).



**Figure 4.3-1 Exemplar standard curve for quantitation of cholesterol**

The standard curve representing the ratio of peak areas (cholesterol/d7-cholesterol) vs. a known amount of cholesterol (µg). A linear regression equation was obtained for standard curve, and line (teal) was deemed acceptable if  $R^2 > 0.98$ . All data are mean  $\pm$  SEM,  $n=3$ , in duplicate. Chol, Cholesterol.



#### 4.3.5. Data Analysis Of Enzyme Kinetics

The percentage inhibition of enzyme velocity by 7-oxysterols was calculated as a ratio of conversion rate in the presence of inhibitor to the conversion rate in the presence of vehicle (ethanol) and multiplied by 100. For calculation of velocity of product generation, non-linear regression analysis of data was performed and the Michaelis-Menten equation applied as described (section 3.3.8, equation 1). Kinetic parameters ( $V_{max}$  and  $K_m$ ) were calculated using Graph Pad prism v5.0.

$K_i$  values were obtained from the Lineweaver-Burke plot or by applying Global Fit Analysis (global nonlinear regression, Graph Pad, Prism v5.0) where an  $R^2$  value =1 (representing the goodness of the fit of the regression lines) indicates competitive inhibition. A Dixon Plot was generated as the reciprocal of the rate of reaction velocity for each substrate concentration  $[S]$  against inhibitor concentration  $[I]$  as follows:

**Equation 1:**  $1/V = (K_m [I]/ V_{max} [S] K_i) + 1/V_{max} (1 + K_m/[S]);$

**Intercept:**  $K_i = K_m/(V_{max}*[S]*slope);$

**Slope** =  $K_m/V_{max}K_i [S];$

The enzyme inhibition constant ( $K_i$ ) was estimated from the graph (e.g. Figure 4.4 – 1) ( $1/V$  vs.  $[I]$ ) using the value for the intercept of the extrapolated regression line on the  $x$ -axis (i.e. when  $y=0$ ,  $[I] = -K_i$ ) or calculated from the slope. Intercepting lines were produced by plotting the data for several substrate concentrations and the point of interception of these lines represents the value of  $K_i$ . The different types of inhibition may be distinguished as follows:

**A competitive inhibitor** – acts only to increase the apparent  $K_m$  for a substrate, the  $V_{max}$  remains unchanged. An increase in  $[S]$  at constant  $[I]$  decreases the degree of

inhibition. The lines of Dixon plot converge above the  $x$ -axis, and the value of  $[I]$  where the lines intersect is  $-1/K_i$ . The intercept on the  $y$ -axis is  $1/V_{\max}$ .

The only effect of a ***non-competitive inhibitor*** is to decrease  $V_{\max}$ . The values of apparent  $K_m$  remain unchanged. The lines of the Dixon plot converge on the  $x$ -axis, and the value of  $[I]$  where the lines intersect is  $-K_i$  (at  $1/V_{\max} = 0$ ). The intercept on the  $y$ -axis is  $1/V_{\max}$  (at  $[I] = 0$ ).

The ***un-competitive inhibitor*** decreases  $V_{\max}$  and  $K_m$  to the same extent. The lines of Dixon plot are parallel.

#### 4.3.6. Statistical Analysis

All data are mean  $\pm$  standard error of the mean (SEM). For purified recombinant m11 $\beta$ HSD1, the means were obtained from three individual experiments. All experiments using animal tissues were carried out using tissues from 6 animals. Each assay was performed in duplicate or triplicate. Statistical significance was determined using Student's unpaired t-test.

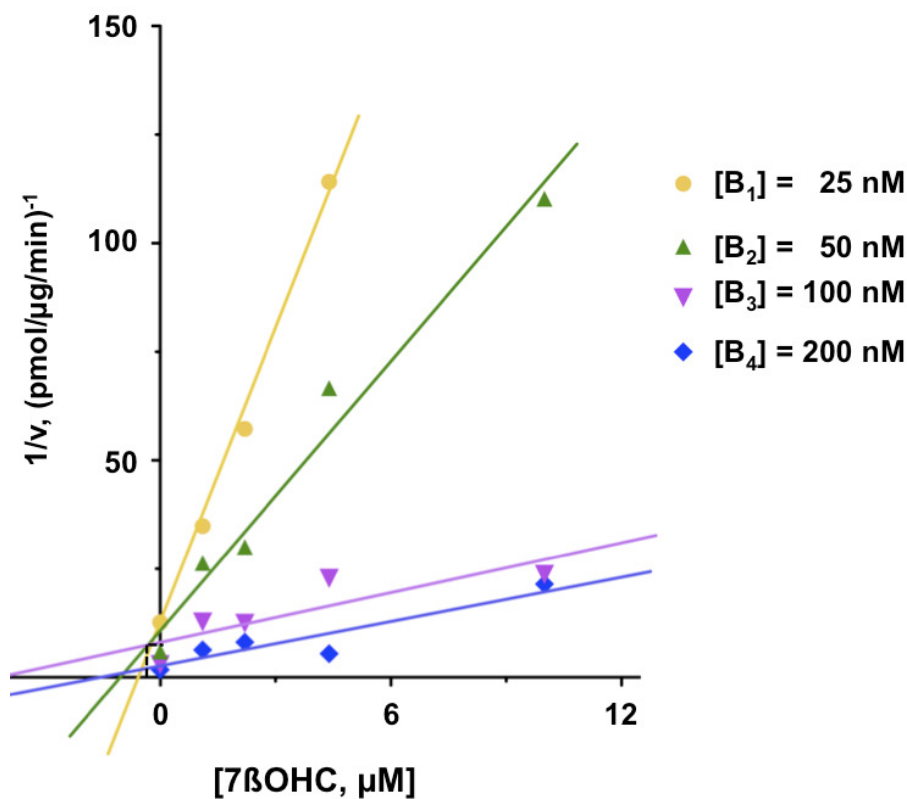
## 4.4. Results

### 4.4.1. Kinetics Of Inhibition Of Dehydrogenation of Glucocorticoids by Oxysterols

The actions of 7-oxysterols to inhibit 11 $\beta$ HSD1-mediated glucocorticoid oxidation were explored using several models. Attempts were also made to investigate the reverse scenario, in which dehydrogenation of 7-oxysterols by 11 $\beta$ HSD1 was assessed in the presence of glucocorticoids (100 nM). However, under these circumstances, the conversion of 7-oxysterols by m11 $\beta$ HSD1 could not be measured in the presence of glucocorticoids, due to stronger binding and faster metabolism of glucocorticoids by 11 $\beta$ HSD1 (as established in section 3.4.3). The presence of the vehicle (ethanol, used to dissolve the inhibitor) did not affect the reaction rate in the amounts used. The presence of 7 $\beta$ OHC or 7-KC did not compromise viability of HEK293 cells, as assessed using trypan blue staining (section 2.6.1.2).

#### 4.4.1.1. *Inhibition of Glucocorticoid Dehydrogenation Catalysed by Recombinant m11 $\beta$ HSD1*

A Dixon Plot was used to identify of the mode of inhibition of glucocorticoid metabolism mediated by oxysterols and for estimation of the dissociation constant (*K<sub>i</sub>*). In Figure 4.4 – 1 a plot of 1/V versus [7 $\beta$ OHC], as the inhibitor, is shown in the presence of substrate (B), thus assessing the potency of 7 $\beta$ OHC to inhibit the oxidation of B. This Dixon plot showed that 7 $\beta$ OHC could competitively inhibit 11 $\beta$ HSD1-mediated inactivation of corticosterone, since the regression lines intercepted above the x-axis. At low physiological substrate concentrations (B, 25-50 nM) the inhibitory effect of 7 $\beta$ OHC was more noticeable than at higher concentrations of B (100 nM), whereas inhibition by 7 $\beta$ OHC was not seen at 200 nM concentration of substrate.



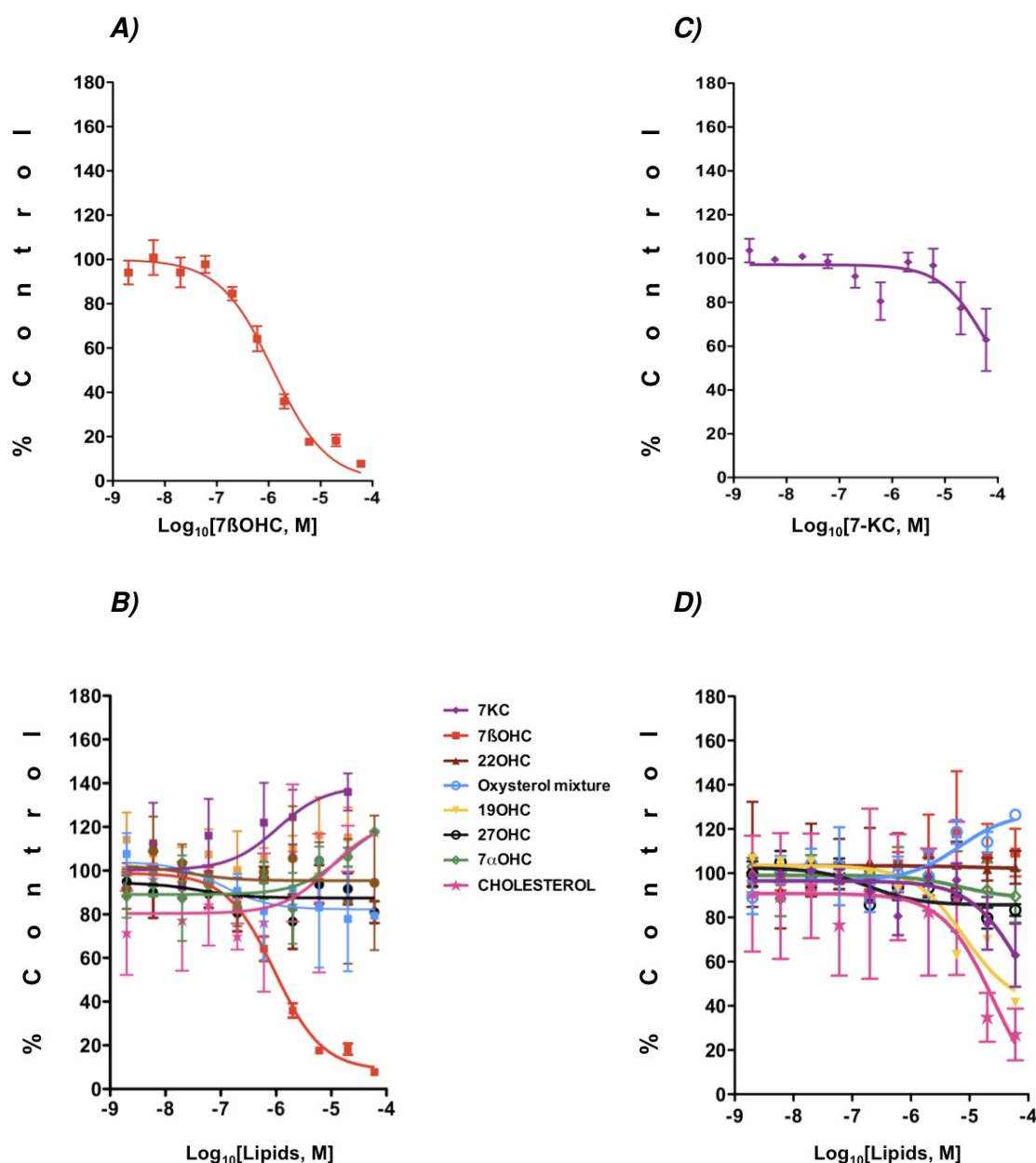
**Figure 4.4-1 Dixon plot of  $1/v$  vs.  $[7\beta\text{OHC}]$  acting to inhibit metabolism of corticosterone to 11-dehydrocorticosterone by recombinant m11 $\beta$ HSD1.**

Conversion of corticosterone (B) to 11-dehydrocorticosterone (A) was quantified in the presence of NADP (2 mM), and 7 $\beta$ -hydroxycholesterol (7 $\beta$ OHC; 1, 2, 4 and 10  $\mu\text{M}$ ). Reaction velocities ( $v$ , pmol/ $\mu\text{g}/\text{min}$ ) were calculated for each concentration of B (25, 50, 100 and 200 nM). A graph of  $1/v$  versus inhibitor (7 $\beta$ OHC) concentration (Dixon Plot) was constructed and resembles the pattern for *competitive inhibition* of corticosterone metabolism. The dissociation constant ( $K_i$ ) for potency of 7 $\beta$ OHC as an inhibitor was determined where the lines on the graph converged above the x-axis (range of B, 25 – 100 nM) and was  $908 \pm 53 \text{ nM}$ . Data are mean, SEM range was  $\pm 3 - 15 \text{ pmol}/\mu\text{g}/\text{min}$  (omitted for clarity of figure),  $n=3$ , in duplicate.

The *K<sub>i</sub>* of 7 $\beta$ OHC was estimated as  $908 \pm 53$  nM. From the intercept on the *y-axis*, the  $1/V_{\max}$  value is obtained, whereby  $V_{\max}$  was estimated as  $0.192 \pm 0.020$  pmol/ $\mu$ g/min and this was unchanged in the presence of inhibitor ( $V_{\max}$   $0.212 \pm 0.008$  pmol/ $\mu$ g/min). The *apparent K<sub>m</sub>* values increased ( $54.5 \pm 2.5 - 178.4 \pm 21.0$  nM) in the presence of inhibitor (25 – 100 nM). Therefore, the Dixon plot resembled *competitive inhibition*.

#### **4.4.1.2. Inhibition Of Glucocorticoid Dehydrogenation Catalysed By m11 $\beta$ HSD1 Expressed In HEK293 Cells.**

The inhibitory potency of 7 $\beta$ OHC was explored in HEK293 cells transfected to generate m11 $\beta$ HSD1. Inhibition of the dehydrogenation of glucocorticoids (B  $\rightarrow$  A) was concentration-dependent and observed at concentrations of 7 $\beta$ OHC as low as 100 nM (Figure 4.4. – 2A). An *IC<sub>50</sub>* of 0.5  $\mu$ M was assigned (Table 4 - 1). These experiments were extended to include other endogenous oxysterols (Figure 4.4. – 2B). Of those tested, 7 $\beta$ OHC was the most potent. Other oxysterols (including 7-KC) did not significantly influence the activity of 11 $\beta$ HSD1, even when tested at concentrations as high as 100  $\mu$ M (Figure 4.4 – 2B).



**Figure 4.4-2 The relative abilities of different oxysterols to inhibit metabolism of glucocorticoids by 11βHSD1.**

The abilities of selected oxysterols to inhibit (**A,C**) dehydrogenation of corticosterone to 11-dehydrocorticosterone and (**B,D**) reduction of 11-dehydrocorticosterone to corticosterone were tested *in vitro*. Percentage inhibition relative to vehicle (% Control) was calculated after treating HEK293 cells expressing m11βHSD1 with the relevant oxysterol diluted in DMEM (1 nM – 10 mM). Data are mean ± SEM from a minimum of n=3-6 experiments performed in duplicate for cholesterol and for each oxysterol (7β-hydroxycholesterol, 7βOHC; 7-ketocholesterol, 7-KC; hydroxycholesterol, OHC).

#### 4.4.1.3. *Inhibition of Dehydrogenation Catalysed By 11 $\beta$ HSD1 in Murine Hepatic Microsomes*

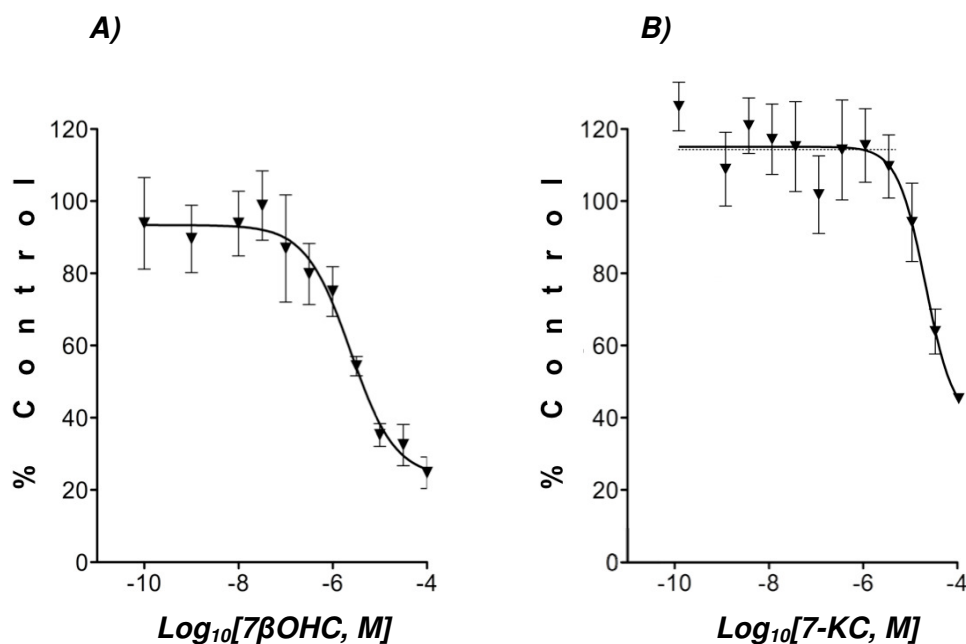
Kinetic parameters describing inhibition of dehydrogenation of glucocorticoids by 7 $\beta$ OHC were further determined in murine hepatic microsomes using various substrate concentrations to determine inhibitory concentrations. The potency of 7 $\beta$ OHC to inhibit metabolism of corticosterone ( $IC_{50}$ ) of 2.2  $\mu$ M was assigned and is summarised in Table 4 - 1 and a graphical example is shown in Figure 4.4 – 3A.

The potency of 7 $\beta$ OHC to inhibit dehydrogenation of corticosterone by microsomal 11 $\beta$ HSD1 was further analysed by constructing Michaelis-Menten and Lineweaver-Burke Plots (Figure 4.4 – 4A & B), yielding a  $K_i = 1.70 \pm 0.06$   $\mu$ M. The maximal velocities ( $V_{max}$ ) remained unchanged in the presence of inhibitor ( $26.01 \pm 9.90$  pmol/mg/min), compared with control ( $35.9 \pm 15.8$  pmol/mg/min). The *apparent*  $K_m$  values increased in the presence of inhibitor ( $951.5 \pm 250$  vs.  $244 \pm 18$  nM in control) resembling *competitive inhibition*.

**Table 4-1  $IC_{50}$  of inhibition of glucocorticoid metabolism by 7 $\beta$ OHC and 7-KC.**

$IC_{50}$ ( $\mu$ M)	HEK293	MLM
	<b>Dehydrogenation of Corticosterone</b>	
<b>7<math>\beta</math>OHC</b>	$0.486 \pm 0.090$	$2.2 \pm 0.4$
<b>7-KC</b>	n/i	n/i
	<b>Reduction of 11-Dehydrocorticosterone</b>	
<b>7<math>\beta</math>OHC</b>	n/i	n/i
<b>7-KC</b>	$35.7 \pm 2.8$	$19.4 \pm 1.2$

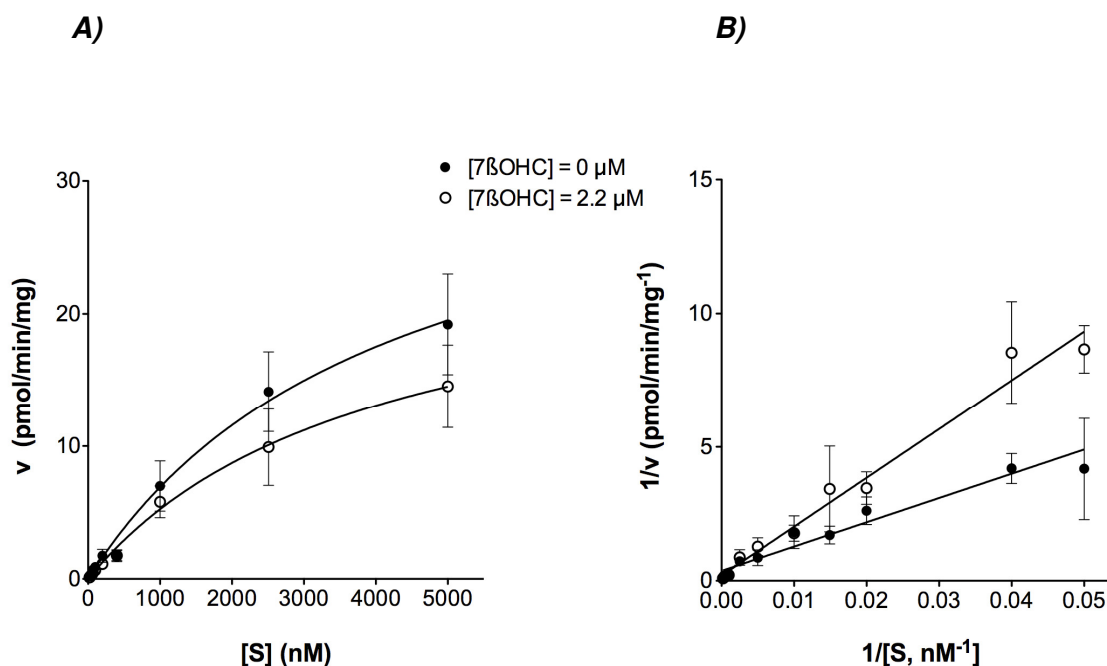
Concentrations ( $IC_{50}$ ) of 7 $\beta$ -hydroxycholesterol (7 $\beta$ OHC) or 7-ketocholesterol (7-KC) resulting in 50% inhibition of metabolism of glucocorticoids by m11 $\beta$ HSD1. The rates of dehydrogenation of corticosterone (B) or reduction of 11-dehydrocorticosterone (A) were measured in HEK293 cells and murine hepatic microsomes (MLM), in the presence of a fixed substrate concentration (20 nM) and a range of concentrations of inhibitor ( $10^{-10}$  -  $10^{-4.5}$  M). The  $IC_{50}$  values for 7 $\beta$ OHC show that it was a better inhibitor of dehydrogenation of B than 7-KC was of reduction of A. Data are mean  $\pm$  SEM, n=6. n/i., inhibition not observed.



**Figure 4.4-3 Inhibition of glucocorticoid metabolism by 7-oxysterols.**

Concentration-dependent inhibition of **A)** dehydrogenation of corticosterone (20 nM) by 7 $\beta$ -hydroxycholesterol (7 $\beta$ OHC;  $10^{-10}$  –  $10^{-4}$  M), and **B)** reduction of 11-dehydrocorticosterone (20 nM) by 7-ketocholesterol (7-KC;  $10^{-10}$  –  $10^{-4}$  M) in murine hepatic microsomes. All data are mean  $\pm$  SEM, n=5, in duplicate.





**Figure 4.4-4 Michaelis-Menten Plot and Lineweaver-Burke transformation of velocities of dehydrogenation of corticosterone by 11βHSD1 in the presence and absence of 7βOHC**

**A)** The velocity of dehydrogenation of corticosterone was assessed in murine hepatic microsomes ( $n = 5 - 6$ ), and presented as a Michaelis-Menten Plot. A range of concentrations of corticosterone was incubated in the presence of vehicle (ethanol; filled circles,) or in the presence of 7βOHC at the previously established  $\text{IC}_{50}$  concentration (2.2  $\mu\text{M}$ ; open circles). **B)** Lineweaver-Burke – double reciprocal plot. A plot of  $1/v$  against  $1/s$  of reaction velocity in the presence of a range of concentrations of corticosterone was compared in the presence of vehicle or 7βOHC. Both Plots show an inhibition of the conversion of B to A by 11βHSD1 in the presence of 7βOHC. All data are mean  $\pm$  SEM,  $n=5$ , in duplicate.

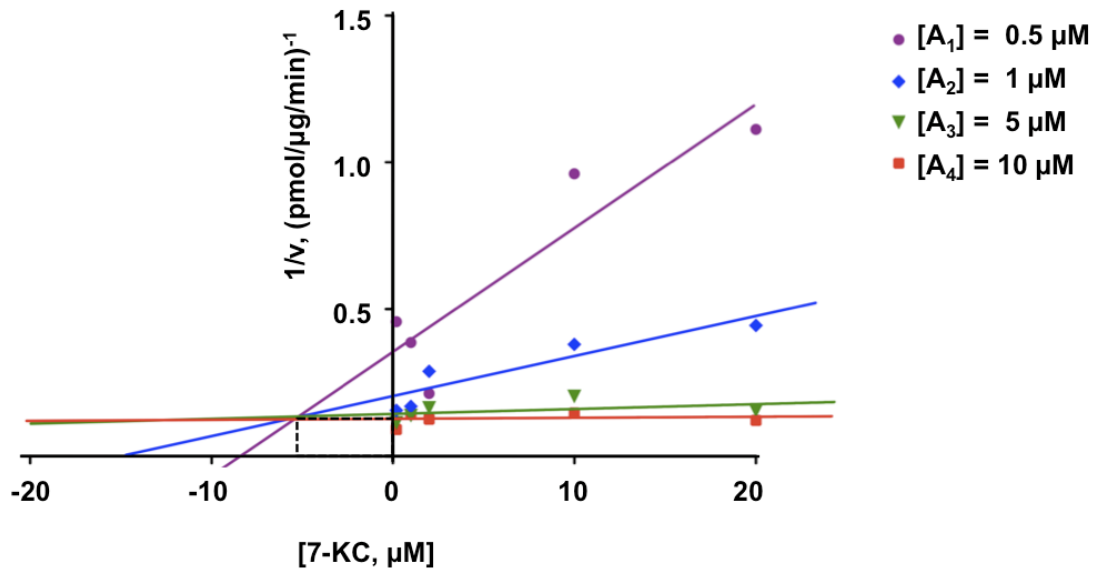
#### 4.4.2. Kinetics Of Inhibition Of Reduction of Glucocorticoids by Oxysterols

##### 4.4.2.1. Inhibition Of Reduction of Glucocorticoids Catalysed By Recombinant m11 $\beta$ HSD1

The kinetics of glucocorticoid metabolism in the presence of 7-oxysterols were explored using Dixon plots. Figure 4.4 – 5 shows a plot of  $1/V$  versus  $[7\text{-KC}]$ , testing the ability of 7-KC to inhibit reduction of 11-dehydrocorticosterone by 11 $\beta$ HSD1. In the presence of the lowest concentration of substrate used (500 nM), 7-KC showed a pronounced inhibition of reduction of A. Its inhibitory actions were diminished with increasing concentrations of A (1  $\mu\text{M}$  – 10  $\mu\text{M}$ ). The lines of  $1/V$  at each substrate concentration converged above the  $x$ -axis and the value for  $K_i$  was estimated as  $7.33 \pm 1.76 \mu\text{M}$ . The  $V_{max}$  was  $6.85 \pm 1.58 \text{ pmol}/\mu\text{g}/\text{min}$  in the presence of inhibitor and this was unchanged from control ( $7.2 \pm 2.0 \text{ pmol}/\mu\text{g}/\text{min}$ ). The *apparent*  $K_m$  values did not increase ( $0.386 \pm 0.020$  –  $0.446 \pm 0.080 \mu\text{M}$ ) in the presence of inhibitor (500 nM – 5  $\mu\text{M}$ ), resembling *a very weak competitive inhibition*.

##### 4.4.2.2. Inhibition of Glucocorticoid Reduction Catalysed by m11 $\beta$ HSD1 expressed in HEK293 cells

7-KC was a *weak inhibitor* of the reduction of 11-dehydrocorticosterone to corticosterone (Figure 4.4 – 2C), achieving only 40% inhibition even at the highest concentration used (100  $\mu\text{M}$ ). Thus an  $\text{IC}_{50}$  was not assigned. Therefore, again in this model, 7 $\beta$ OHC was a better inhibitor of dehydrogenation (B $\rightarrow$ A) than 7-KC was of reduction (A $\rightarrow$ B). Again these experiments were extended to include other oxysterols (including 7 $\beta$ OHC), none of which inhibited reduction of A to B, although cholesterol alone exhibited some inhibitory effects (Figure 4.4 – 2D).



**Figure 4.4-5 Dixon plot of  $1/v$  vs. [7-KC] acting to inhibit reduction of 11-dehydrocorticosterone to corticosterone by recombinant m11 $\beta$ HSD1.**

The velocity of reduction of 11-dehydrocorticosterone (A) to corticosterone (B) was quantified in the presence of NADPH (2mM), and 7-ketocholesterol (7-KC) as an inhibitor (500 nM, 1, 2, 10 and 20  $\mu\text{M}$ ). Reaction velocities ( $v$ , pmol/ $\mu\text{g}/\text{min}$ ) were calculated for each concentration of A (500 nM, 1, 5 and 10  $\mu\text{M}$ ). A graph of  $1/v$  versus inhibitor (7-KC) concentration (Dixon Plot) was constructed and resembles the pattern for *competitive inhibition* of 11-dehydrocorticosterone metabolism. The dissociation constant ( $K_i$ ) for potency of 7-KC as an inhibitor was determined where the lines on the graph converged above the  $x$ -axis (range of A, 0.5 - 1  $\mu\text{M}$ ) and was  $7.3 \pm 1.7 \mu\text{M}$ . 7-KC. Data are mean; SEM range was  $\pm 0.02 - 0.3$  pmol/ $\mu\text{g}/\text{min}$  (omitted for clarity of figure),  $n=3$ , in duplicate.

#### 4.4.2.3. *Inhibition of Reduction Catalysed by 11 $\beta$ HSD1 in Murine Hepatic Microsomes*

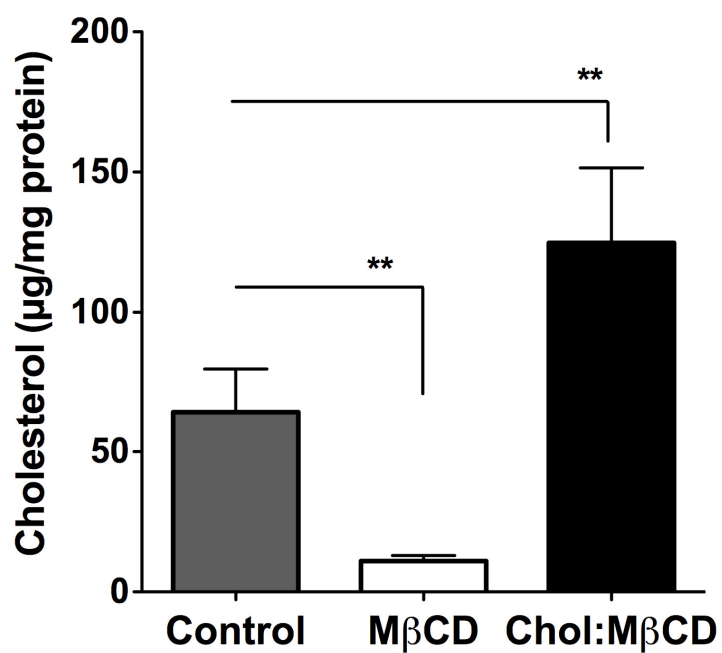
The ability of 7-KC to inhibit metabolism of 11-dehydrocorticosterone ( $IC_{50}$ ) is summarised in Table 4 -1 and a graphical example is shown in Figure 4.4 – 3B. 7-KC was a *weak inhibitor* of the reduction of 11-dehydrocorticosterone, and further analysis (Michaelis-Menten and Lineweaver-Burke) of its potency to inhibit this reaction by microsomal 11 $\beta$ HSD1 was not conducted. Thus in this model as well, 7 $\beta$ OHC was a better inhibitor of dehydrogenation (B $\rightarrow$ A) than 7-KC was of reduction (A $\rightarrow$ B).

#### 4.4.3. *Cholesterol Depletion In Vitro*

Incubating HEK293 cells stably-transfected to produce m11 $\beta$ HSD1, with culture medium containing M $\beta$ CD resulted in a reduction of cholesterol levels. Total levels of cholesterol were reduced by ~87% within 1h of treatment compared to controls (Figure 4.4 – 6).

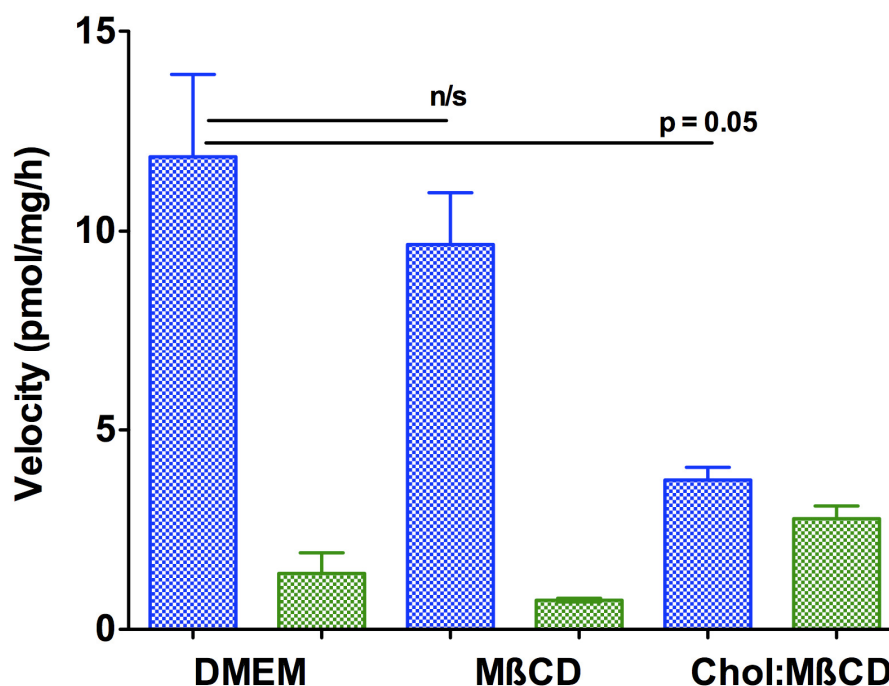
Cholesterol depletion reduced the levels of 7-oxysterol in HEK293 cells and in some cases these levels were undetectable (Limit of detection (LOD)  $3.2 \pm 0.2$  pmol/mg). Basal levels of 7-oxysterols were always detected in control cells (DMEM only), in the following ranges, **7-KC**:  $19.40 \pm 1.08$  pmol/mg and **7 $\beta$ OHC**:  $4.37 \pm 1.90$  pmol/mg. These levels were within the range reported for the intact cells treated with vehicle only (Chapter 3).

The ability of 11 $\beta$ HSD1 to convert steroids in the cells was monitored, in both the reductase and dehydrogenase directions upon cellular depletion of cholesterol with M $\beta$ CD. As anticipated, in controls the conversion of 11-dehydrocorticosterone to corticosterone by 11 $\beta$ HSD1 predominated over the reverse reaction. Interestingly, the depletion of cellular cholesterol or 7-oxysterol levels in HEK293 did not influence 11 $\beta$ HSD1 activity, in either the reductase or dehydrogenase directions (Figure 4.4 – 7).



**Figure 4.4-6 Cholesterol manipulation *in vitro*.**

HEK293 cells expressing m11βHSD1 were depleted of cholesterol using methyl-β-cyclodextrin carrier (MβCD, 10 mM), while upload of cholesterol was achieved using a complex of cholesterol with the MβCD carrier (Chol:MβCD, 1:6, 5 mM). The levels of cholesterol in the cells were measured using gas chromatography/mass spectrometry (GC/MS). All data are mean ± SEM, n=4, in duplicate, expressed per milligram protein. Differences were assessed using 1-way ANOVA, \*\*p<0.01.



**Figure 4.4-7 The influence of cellular cholesterol content on the velocity of glucocorticoid metabolism by m11βHSD1.**

HEK293 cells stably-transfected to express m11βHSD1 were treated with DMEM containing methyl-β-cyclodextrin (MβCD, 10 mM, 1h) or cholesterol in a complex with MβCD (Chol:MβCD, 1:6 ratio, 1h). Control cells were treated with culture medium only (DMEM). After manipulation of cholesterol, the reaction velocity was measured in the reductase (red, A → B) and dehydrogenase (blue, B → A) directions. The reaction velocities were expressed as pmol/mg protein/h. Incubation of cells with the cholesterol lowering complex, MβCD, depleted cells of cholesterol (Figure 4.4 - 6) but did not affect directionality of 11βHSD1. Incubation of HEK293 cells with cholesterol in complex with MβCD (Chol: MβCD), resulted in cholesterol loading. This treatment significantly lowered the rate of reduction of A → B and there was a trend for an increase in velocity of dehydrogenation of B → A (p=0.09). Thus cholesterol loading, but not depletion, alters the set-point of the equilibrium of 11βHSD1. All data are mean ± SEM, n=4, in duplicate. Differences were assessed using unpaired Student's t-test. DMEM, Dulbecco's modified Eagle's medium.

#### 4.4.4. Cholesterol Loading - Cholesterol:M $\beta$ CD Complex

Incubation of HEK293 cells with cholesterol complexed with M $\beta$ CD resulted in a significant increase ( $p < 0.01$ ) in cholesterol levels compared with controls (Figure 4.4 – 6). To test that this treatment was reversible, cells loaded with cholesterol were then treated with M $\beta$ CD, and this successfully lowered the cholesterol levels ( $105.6 \pm 9$ ;  $**p < 0.01$  vs. loading) within 30 min or returned them to those of control levels ( $78.4 \pm 12$   $\mu$ g/mg) within 1h of treatment.

The levels of 7-oxysterols were more readily detected after cholesterol loading, and levels of 7-KC were significantly higher compared with control (7-KC:  $39.48 \pm 3.01$  vs.  $19.40 \pm 1.08$  pmol/mg;  $p < 0.05$ ), while in the case of 7 $\beta$ OHC there was a trend ( $p = 0.08$ ) towards increased concentrations (7 $\beta$ OHC:  $17.6 \pm 2.4$  pmol/mg vs. undetected, LOD  $3.2 \pm 0.2$  pmol/mg) compared with control.

Following cholesterol loading, the velocity of reduction of 11-dehydrocorticosterone to corticosterone was significantly reduced (Figure 4.4 – 7), compared with control and there was a trend for an increased rate of dehydrogenation of corticosterone.

## 4.5. Discussion

The work presented in this chapter tested the hypothesis that the presence of 7-oxysterols influences the metabolism of glucocorticoids by 11 $\beta$ HSD1. The initial aim of this work was to investigate the competition for 11 $\beta$ HSD1 metabolism by the two classes of substrates, glucocorticoids and 7-oxysterols. Subsequently the influence of *in vitro* manipulation of cellular cholesterol levels on metabolism of glucocorticoids by 11 $\beta$ HSD1 was investigated.

### 4.5.1. 7-Oxysterols Compete With Glucocorticoids For Metabolism By m11 $\beta$ HSD1

Kinetic parameters determined in this chapter indicate that 7-oxysterols not only act as substrates for 11 $\beta$ HSD1 but also compete with glucocorticoids (the conventional substrates) for metabolism by this enzyme. Three different approaches were taken to test the competition between 7-oxysterols and glucocorticoids for metabolism by m11 $\beta$ HSD1, using: recombinant m11 $\beta$ HSD1 protein, murine hepatic microsomes and intact (HEK293) cells stably expressing m11 $\beta$ HSD1. Two different approaches were used to assess the *K<sub>i</sub>* for binding of each 7-oxysterol; Lineweaver-Burk and Dixon plot (both linearised representations of the Michaelis-Menten equation). It has been shown before that Dixon plots do not provide a more robust estimate of *K<sub>i</sub>* than Lineweaver-Burk plots (Kakkar *et al.*, 1999) and the values obtained between the two approaches were certainly comparable. However, Dixon plots were used in order to establish the type of inhibition between the two classes of substrates.

The estimates of *K<sub>i</sub>* were important as they predict whether the inhibitory actions of 7-oxysterols on glucocorticoids are likely to occur *in vivo*, taking into account physiological levels of these substrates. The range of inhibitory concentrations of 7-oxysterols used in this work, encompassed the physiological range of 7-oxysterols present in human plasma (low nM) and the pathophysiological levels (low  $\mu$ M) detected in atherosclerotic plaques (Brown *et al.*, 1997; Dzeletovic *et al.*, 1995a; Dzeletovic *et al.*, 1995b; Prunet *et al.*, 2006). The inhibitory concentrations



obtained using all three approaches described above have predicted that concentrations of 7-oxysterols in a range between 900 nM and 8  $\mu$ M may have potent inhibitory actions on metabolism of glucocorticoids by 11 $\beta$ HSD1 in the body.

Interestingly, the work in this chapter also demonstrates that 7-oxysterols, but not other oxysterols, inhibit glucocorticoid metabolism, at least in HEK293 cells. Previous investigations (Biasi *et al.*, 2004; Steffen *et al.*, 2006) have mostly studied the effect of individual oxysterols on cell viability. However, oxysterols may have synergistic effects that exacerbate pro-apoptotic actions suggesting that combinations of different oxysterols should be applied to these models (Lizard, 2006). Such conditions were investigated in the work described here, but a mixture of oxysterols did not display the ability to inhibit the metabolism of glucocorticoids by 11 $\beta$ HSD1. However, this situation in other cells, particularly during disease pathogenesis may be more complex: for example, during macrophage accumulation in atherosclerotic plaque development. It would be anticipated that macrophages might accumulate oxysterols, which could further promote apoptosis of these cells as well as inhibit the 11 $\beta$ HSD1-mediated conversion of glucocorticoids (Hadoke *et al.*, 2009).

Consistently in each approach, the inhibitory effects of 7-oxysterols on glucocorticoid metabolism were concentration-dependent and reached half maximal inhibitory concentrations ( $IC_{50}$ ) in concentrations ranging from 0.5 – 20  $\mu$ M. 7-KC and 7 $\beta$ OHC acted in opposite ways, with 7-KC slowing the rate of reduction but 7 $\beta$ OHC diminishing dehydrogenation catalysed by 11 $\beta$ HSD1. The effects of 7 $\beta$ OHC were more pronounced, particularly at lower concentrations of glucocorticoids. The kinetic measurements showed that 7 $\beta$ OHC was a strong *competitive inhibitor* of dehydrogenation of corticosterone but it did not inhibit the reduction of 11-dehydrocorticosterone. One interpretation of this finding is that inhibitory actions of 7 $\beta$ OHC may act to predominantly slow dehydrogenation of corticosterone, thereby sustaining the predominant generation of active glucocorticoid via reduction of 11-dehydrocorticosterone, in rodents.

Based on the demonstration that both 7-KC and 11-dehydrocorticosterone can be metabolised by 11 $\beta$ HSD1, it was expected that 7-KC would act as a potent *competitive inhibitor* of 11 $\beta$ HSD1 reductase activity. However, although this effect could be seen in all model systems, 7-KC was consistently less effective as an inhibitor of reduction, compared with 7 $\beta$ OHC acting to prevent dehydrogenation. During preparation of this thesis a paper by Balázs *et al.* (2009) reported that they could not detect any inhibition of human 11 $\beta$ HSD1 reductase activity in lysates or HEK293 cells by 7-KC or 7 $\beta$ OHC. Even in HEK293 cells stably expressing 11 $\beta$ HSD1 and H6PDH (Dakin, 2008), inhibition of reductase activity by 7-KC was not observed. However this is not the case in all cells tested. Balázs *et al.* have reported only the inhibition of 11 $\beta$ HSD1 reductase activity by 7-KC (IC<sub>50</sub> 8.1  $\pm$  0.9  $\mu$ M) in differentiated THP-1 macrophages, and half as efficiently by 7 $\beta$ OHC (of *reductase*), whereas Wamil *et al.*, (2009) have shown inhibitory actions of 7-KC on reductive metabolism by 11 $\beta$ HSD1 in 3T3-L1 human adipocytes. It is unclear why 11 $\beta$ HSD1 activity is differentially affected by 7-KC or 7 $\beta$ OHC but perhaps this effect is cell type dependent. It is possible that cell-specific inhibition by 7-KC is due to differential post-transcriptional modification of 11 $\beta$ HSD1 *in vivo* (such as phosphorylation, or glycosylation at asparagine<sup>207</sup> (Asn<sup>207</sup>)) which are known to be essential for enzyme activity in either direction (Agarwal *et al.*, 1995). Such post-transcriptional modification may not affect the binding or catalysis of glucocorticoids, but may interfere with the binding of 7-oxysterols. Another indication that the ability of 7-oxysterols to inhibit 11 $\beta$ HSD1-mediated GC metabolism is cell-specific is provided by the fact that some cells accumulate 7-oxysterols more readily than others (a characteristic example being adipocytes and macrophages (Brown and Jessup, 1999, 2009)). However, overall the findings presented in this Chapter are generally consistent with those in Ch3, in that 7-KC is less able to bind efficiently to the active site of 11 $\beta$ HSD1.

The converse argument may also be proposed, that glucocorticoids inhibit metabolism of 7-oxysterols by 11 $\beta$ HSD1. Indeed, this possibility has been raised previously in relation to non-glucocorticoid substrates by Robinson *et al.*, (2003). This has been briefly explored in this thesis, but conversion of 7-oxysterols by

11 $\beta$ HSD1 could not be measured in the presence of 100 nM glucocorticoids. It appears that, due to lower rates of inter-conversion of 7-oxysterols, the presence of glucocorticoids can overcome the ability of 11 $\beta$ HSD1 to metabolise 7-oxysterols (as established in section 3.4.3) and the same phenomenon is likely to happen *in vivo*.

It is likely that, under conditions of low circulating glucocorticoids, 7-oxysterols will have greater potential to inhibit 11 $\beta$ HSD1-mediated metabolism of glucocorticoids (as elucidated by the Dixon plot analysis). In contrast, the inhibitory potency of 7-oxysterols is likely to be attenuated when glucocorticoid levels increase e.g. in the morning or under stressful circumstances. It is, therefore, of interest to assess the levels of 7-KC and 7 $\beta$ OHC concomitantly with those of corticosterone and 11-dehydrocorticosterone when attempting to interpret the likely role of 11 $\beta$ HSD1 in physiology or pathophysiology.

Furthermore, it may be proposed that oxysterol-mediated competitive inhibition of reduction of glucocorticoids will be more significant under hyperlipidaemic conditions (which are associated with a pathological increase of oxidised lipids in tissues and plasma, (Brown and Jessup, 2009)) than in normolipidaemia (Wamil *et al.*, 2008). For example, inhibition of this reaction may occur in peripheral tissues, such as the aortae and macrophages, in which oxysterols can accumulate and reach micromolar concentrations (Brown and Jessup, 1999). Although high levels of cholesterol are precursors for increased synthesis of 7-oxysterols via non-enzymatic routes, it is clear that generation by 11 $\beta$ HSD1 may also affect their local levels (Jessup and Brown, 2005). However, all potential interactions that may happen between 7-oxysterols and glucocorticoids *in vivo* are predicated on the assumption that 7-oxysterols are transported across the ER membrane (Massey, 2006) to the site of 11 $\beta$ HSD1 activity.

#### **4.5.2. Manipulation Of Cellular Cholesterol Levels**

In view of the above findings it is possible that factors influencing synthesis of 7-oxysterols may alter the kinetics of metabolism by 11 $\beta$ HSD1. It seems logical that increased cholesterol levels *in vivo* would contribute to subsequent increases in

oxysterol levels but this relationship is poorly understood. This concept was explored further in the context of variations in cellular concentrations of cholesterol. Methyl- $\beta$ -cyclodextrin (M $\beta$ CD) was used to acutely deplete cholesterol in cultured cells, where it acted as a carrier to bind to and remove cholesterol from the membranes. It has been suggested that M $\beta$ CD binds cholesterol in lipid rafts (Christian *et al.*, 1997; Gaus *et al.*, 2004; Kilsdonk *et al.*, 1995). Lipid rafts are membrane microdomains involved in many cellular functions, including transduction of cellular signals and cell entry by pathogens (Zeng *et al.*, 2009).

In addition, incubation of M $\beta$ CD with high concentrations of cholesterol can form a saturated complex (Chol:M $\beta$ CD) which is used *in vitro* to deliver cholesterol to the cellular membranes by active transport. This complex has been used at a 1:8 molar ratio (i.e. the saturation limit of M $\beta$ CD) (Danthi *et al.*, 2004, Klein *et al.*, 1995), but in practice in this work, a 1:6 mix was found most efficient. Using cholesterol depletion/loading approaches, the work in this chapter demonstrates that depletion of cellular cholesterol levels leads to a subsequent reduction in the 7-oxysterol levels, whereas increase of cholesterol leads to an increase in 7-oxysterol levels. Most importantly, these findings prove that cholesterol is the primary source of some 7-oxysterols in the cell-type tested.

The depletion of cholesterol in HEK293 cells showed that, despite a reduction in the intra-cellular levels of 7-oxysterols, the rate of 11 $\beta$ HSD1-mediated glucocorticoid metabolism remained similar to controls. A concern with the experimental model was that depletion of cholesterol (which, if prolonged, may potentially lead to disruption of cell membranes (Byfield *et al.*, 2004)), might also have disturbed the coupling between 11 $\beta$ HSD1 and H6PDH (Bujalska *et al.*, 2008; Hewitt *et al.*, 2005). However, upon removal of cellular cholesterol the activity of 11 $\beta$ HSD1 remained unchanged. Therefore this suggests that cholesterol depletion did not affect the orientation of 11 $\beta$ HSD1 in the membrane.

Loading the cells with cholesterol simulated the scenario in obesity and dyslipidaemia, where high concentrations of cholesterol may promote high

concentrations of oxysterols within the oxLDL. Therefore, based on previous findings (section 4.5.1), it was hypothesized that increased levels of 7 $\beta$ OHC in oxLDL would inhibit dehydrogenation of glucocorticoids by 11 $\beta$ HSD1, thus promoting reduction of inactive to an active glucocorticoid by 11 $\beta$ HSD1. Loading cholesterol into HEK293 cells, using a cholesterol:M $\beta$ CD complex, successfully increased the levels of 7-oxysterols. However, the effect on enzyme direction was opposite to that proposed, in that reduction decreased and dehydrogenation increased. It is possible this is a consequence of the increase in cellular cholesterol, since the data in Section 4.4.2 show that this lipid could inhibit reduction independently.

It is again possible that loading HEK293 cells with cholesterol, might have altered distribution of lipids in the cell membrane. This would cause disruption to the cell membrane, which could be predicted to alter the stability of 11 $\beta$ HSD1 within the ER and thus influence its activity. Indeed disruption of the cellular environment has been shown to suppress reduction and promote dehydrogenation of glucocorticoids by 11 $\beta$ HSD1 in tissues *ex vivo* (Bujalska *et al.*, 2005; Bujalska *et al.*, 2002), consistent with the data in this chapter. Recent work however, with lipid-rich THP-1 macrophages or adipocytes (Balázs *et al.*, 2009; Wamil *et al.*, 2008), associated the ability of 7-KC to inhibit 11 $\beta$ HSD1 reductase activity (dehydrogenase was not studied) with the ability of these cells to sequester oxysterols. This suggests therefore that the effects of cholesterol loading differ in cells that physiologically accumulate lipids (e.g. macrophages and adipocytes) and those that are not (e.g. VSMC). To shed further light on this relationship, future experiments could be performed using primary macrophages (expressing 11 $\beta$ HSD1) from hyperlipidaemic or atherosclerotic mice, (Gilmour and *et al.*, 2006), where accumulation of cholesterol (Qin *et al.*, 2006) and 7-oxysterols has been demonstrated (Brown and Jessup, 1999; Libby, 2002a; Lizard *et al.*, 2006). Although primary macrophages and adipocytes are the candidate cells for this work, due to expression of 11 $\beta$ HSD1, it is crucial to consider that 7-oxysterols may readily be utilised *via* other enzymes present in these

cells, such as 27-hydroxylase (Javitt, 2008), making these systems more complex to study.

Certainly, all of the findings in this Chapter have implications in obesity and hypercholesterolaemia, as they suggest that long-term accumulation of lipids in cells will influence activation of glucocorticoids by 11 $\beta$ HSD1; this remains to be confirmed *in vivo*. In relation to pathophysiology, pharmacological inhibition of 11 $\beta$ HSD1 in atherosclerosis is beneficial as it reduces the actions of active glucocorticoid (Hermanowski-Vosatka *et al.*, 2005). The data in this chapter show that beneficial effects of inhibition of 11 $\beta$ HSD1 may also be due to further interactions with 7-oxysterols.

## **Chapter 5. Levels Of 7-Oxysterols In Murine Plasma And Tissues**

## 5.1. Introduction

Oxysterols are present in the circulation at far greater concentrations than cholesterol. The proportions of different oxysterols can vary between plasma lipoprotein fractions (Babiker and Diczfalusy, 1998; Diczfalusy *et al.*, 1996) and in tissues (Griffiths *et al.*, 2006; Leoni *et al.*, 2005; Tsai, 1984; Vatassery *et al.*, 1997; Yoshida *et al.*, 2003). In healthy humans, 7-oxysterols circulate in low micromolar concentrations (0.15 – 0.73  $\mu\text{M}$ ), of which ~10% constitutes the un-esterified form (Dzeletovic *et al.*, 1995b). Under pathological conditions, the levels of particular oxysterols may increase by up to 100 times (Adachi *et al.*, 2000; Katz *et al.*, 1982).

Findings from experiments described in previous chapters of this thesis have indicated that cellular levels of 7-KC and 7 $\beta$ OHC may be dependent on the activity of 11 $\beta$ HSD1. The influence of 11 $\beta$ HSD1 on circulating and tissue levels of oxysterols has not been extensively explored. It has been proposed that 11 $\beta$ HSD1 may play a role in detoxification of accumulated 7-KC by preferentially converting 7-KC to 7 $\beta$ OHC in the liver (Schweizer *et al.*, 2004). The inhibition of this enzyme (using carbenoxolone) caused a hepatic accumulation of 7-KC in rats (Schweizer *et al.*, 2004). In a more recent study, Larsson *et al.*, (2007) showed a rapid inter-conversion of circulating, deuterium-labelled 7-KC and 7 $\beta$ OHC, in healthy volunteers. They speculated that changes in circulatory levels of both 7-oxysterols could be assigned to the actions of 11 $\beta$ HSD1. The results from chapter 3 proposed a dominant dehydrogenation activity of 11 $\beta$ HSD1 (7 $\beta$ OHC to 7-KC) in cells, which may lead to accumulation of 7-KC. Although, the systems assessed in previous chapters may not represent actual *in vivo* conditions for measuring the activity of 11 $\beta$ HSD1, it still remains possible that 11 $\beta$ HSD1 acts as a reductase *in vivo* (7-KC to 7 $\beta$ OHC). Therefore, inhibition of 11 $\beta$ HSD1 would lead to accumulation of 7-KC and reduction in 7 $\beta$ OHC.

Several research groups have reported increased levels of 7-oxysterols in the plasma of patients with atherosclerosis and diabetes mellitus type 2 (0.1 - 0.6  $\mu\text{M}$ ), in comparison with normal subjects (low nM, 10 –30 nM) (Endo *et al.*, 2008). In foam



cells, atherosclerotic plaques and hyperlipidaemic serum levels of 7-oxysterols can reach high micromolar concentrations (Brown et al., 2000a; Brown and Jessup, 1999; Hodis et al., 1991; Hodis et al., 2000). The accumulation of 7-KC and 7 $\beta$ OHC in the plasma of patients with hyperlipidaemia and diabetes mellitus is suggested as a biomarker of oxidative stress (Arca *et al.*, 2007; Ferderbar *et al.*, 2007; Katsumi *et al.*, 2000; Zwirska-Korczala *et al.*, 2002). 11 $\beta$ HSD1 may also be regulating the proportion of 7-KC to 7 $\beta$ OHC, as the ratio of these compounds seems to be important for the final (patho)physiological output (Steffen *et al.*, 2006). Despite contributing to an *in vivo* clearance of 7-oxysterols, it is still not understood if 11 $\beta$ HSD1 is regulating their plasma or cellular concentrations. Given that 7-oxysterols may have a distinct function in the body and may inhibit glucocorticoid metabolism, it is essential to clarify the contribution of 11 $\beta$ HSD1 to the levels of 7-oxysterols in circulation. Further, the regulation of circulatory 7-oxysterols by 11 $\beta$ HSD1 is of importance for understanding the atheroprotective effect of 11 $\beta$ HSD1 inhibition.

## 5.2. Hypothesis And Aims

The hypotheses underlying the work presented in this chapter were that 11 $\beta$ HSD1 mediates the ratios of circulating 7-oxysterols, and that 7-KC would accumulate in the plasma and tissues upon inhibition of 11 $\beta$ HSD1.

Therefore, the specific aims were to:

- Determine the levels of 7-oxysterols in normolipidemic mice by adapting current methods used in humans;
- Investigate whether 11 $\beta$ HSD1 disruption in mice decreases the ratio of 7 $\beta$ OHC to 7-KC, in that it will reduce the levels of 7 $\beta$ OHC and/or increase levels of 7-KC in plasma and tissues.
- Determine whether levels of 7-oxysterols are elevated in plasma and tissues of atherosclerotic (ApoE<sup>-/-</sup>) mice, and
- Investigate whether 11 $\beta$ HSD1 inhibition in ApoE<sup>-/-</sup> mice decreases the ratio of 7 $\beta$ OHC to 7-KC.

### 5.3. Methods

Method development was performed using plasma from a readily available source (5-alpha reductase-1 heterozygous; 5 $\alpha$ SRD1<sup>+/-</sup> mice). The concentrations of cholesterol and oxysterols were quantified in plasma, livers and/or aortae from three murine models (C57Bl/6, ApoE<sup>-/-</sup> and 11 $\beta$ HSD1<sup>-/-</sup>).

#### 5.3.1. Method Development

In order to establish the conditions for gas chromatography/ mass spectrometry (GC/MS), preliminary work was performed to adapt methods from the literature for use with murine plasma. For this purpose plasma from surplus, male and female, 5-alpha reductase-1 heterozygous (5 $\alpha$ SRD1<sup>+/-</sup>) mice (age 1 year, n=12) was used (Mahendroo *et al.*, 2001). Initial work aimed to determine the sensitivity of methods found in the literature and to establish the minimum volume of murine plasma needed for determining the levels of 7-oxysterols. The process of method development is described in section 5.4.1. Plasma (400-500  $\mu$ l) was collected by pooling samples to obtain n=6 per gender group. Levels of 7-oxysterols were determined following lipid extraction and derivatisation steps (section 2.11.1 and 2.13.2) and the amount of cholesterol and 7-oxysterols quantified using GC/MS (section 2.13.3).

#### 5.3.2. Does Disruption Of 11 $\beta$ HSD1 in Mice Regulate The Levels Of 7-Oxysterols?

C57Bl/6 and 11 $\beta$ HSD1<sup>-/-</sup> mice (male, age 20 weeks, n=10) were killed and plasma and livers (section 2.4) obtained and processed (section 5.3.4). Plasma (400-500  $\mu$ l) was collected by pooling samples. Further extractions of lipids from plasma and hepatic fractions (100-200  $\mu$ l) were performed as described (see sections 2.11.1 - 3). Levels of 7-oxysterols were determined as above (section 5.3.1). What are the Levels Of 7-Oxysterol in Atherosclerotic ApoE<sup>-/-</sup> Mice?

ApoE<sup>-/-</sup> mice (male, age 22 weeks, n=6) were fed an atherogenic Western diet (D12079B, Research diets, USA; fat 21%, protein 29%, carbohydrate 50%, cholesterol 0.21%) for 14 weeks. Plasma (250-400  $\mu$ l) was collected from individual

animals. Levels of 7-oxysterols and cholesterol were determined as above (section 5.3.1)

### **5.3.3. Does Inhibition Of 11 $\beta$ HSD1 Influence The Levels Of 7-Oxysterols In Atherosclerotic ApoE<sup>-/-</sup> Mice?**

ApoE<sup>-/-</sup> mice (male, age 8 weeks, n=6) were fed an atherogenic Western diet for 6 weeks and then randomized to receive either Western diet (as in 5.3.3, controls) or an 11 $\beta$ HSD1 inhibitor (10 mg/kg/day, mixed within Western diet) for a further 8 weeks. The 11 $\beta$ HSD1 inhibitor used was the Merck compound 544 ((3-(1-adamantyl)-6,7,8,9-tetrahydro-5H-[1,2,4]triazolo[4,3-a]azepine; Enamine Ltd., Ukraine), at a dose which has previously been shown to inhibit cholesterol accumulation in the aortae of ApoE<sup>-/-</sup> mice (Hermanowski-Vosatka *et al.*, 2005). Levels of 7-oxysterols and cholesterol were determined as above (section 5.3.1).

### **5.3.4. Tissue Handling At Cull**

All mice were culled by decapitation, except ApoE<sup>-/-</sup> mice (section 5.3.4.1), which were culled by asphyxiation. Plasma samples were obtained as described (section 2.4.6). Hepatic microsomal and cytosolic fractions were obtained as in section 2.4.2. Aortae were processed (as in section 2.4.5) and protein concentrations were determined in hepatic and aortic preparations (section 2.4.7). 7-oxysterols were extracted from plasma (300-500  $\mu$ l), microsomes (100  $\mu$ l) and cytosols (200  $\mu$ l) as described (section 2.11.1) and levels quantified as before (section 2.13.3).

### **5.3.5. Evaluation Of Efficacy Of 11 $\beta$ HSD1 Inhibitor**

#### **5.3.5.1. Inhibition Of 11 $\beta$ HSD1 Activity In Vitro**

For *in vitro* studies, HEK293 cells transfected with murine 11 $\beta$ HSD1 (m11 $\beta$ HSD1) were treated with steroids (30 nM) or 7-oxysterols (1  $\mu$ M) as described (section 2.6). The 11 $\beta$ HSD1 inhibitor (1  $\mu$ M) was dissolved in water that contained 2-hydroxypropyl- $\beta$ -cyclodextrin (5%, w/v) and vortexed (400 rpm, 2 min). Cells were treated with inhibitor or vehicle (5% w/v, 2-hydroxypropyl- $\beta$ -cyclodextrin in water) for 2 hours prior to addition of medium containing steroids or oxysterols, and incubated for a further 35 min or 16 hours, respectively. All *in vitro* assays of steroid conversion were performed in 12-well plates, whilst all assays of conversion of 7-oxysterols were performed in 6-well plates. The control wells contained medium with vehicle (5%, w/v) and no cells, to which EtOH, 7-oxysterols or steroids were added.

#### **5.3.5.2. Inhibition Of 11 $\beta$ HSD1 Activity Ex-Vivo**

Assays of inhibition of inter-conversion of corticosterone (B) to 11-dehydrocorticosterone (A) by 11 $\beta$ HSD1 in hepatic homogenates were performed as described (section 2.7.1), except that the concentration of unlabelled B was 25 nM. Assays of 11-dehydrocorticosterone (A) conversion to corticosterone (B) by 11 $\beta$ HSD1 in hepatic homogenates were performed as described (section 2.7.3), except that the concentration of unlabelled A was 25 nM. The low concentrations were employed to enhance the possibility of revealing inhibition *ex vivo*. Samples were processed as described (sections 2.7.1 and 2.7.3), and detection of radiolabelled steroids performed (section 2.12.2).

### **5.3.6. Extraction Of Oxysterols And Cholesterols From Biological Fluids And Tissue**

Cholesterol and oxysterols were extracted from plasma (minimum volume 300  $\mu$ l) and biological tissue together with d7-standards, as described in the final optimized

method (sections 2.11.1 and 2.11.2). The extraction and processing of sub-cellular fractions (200 µl) was performed as described (section 2.11.3). Oxysterols and cholesterol were analysed and detected using GC/MS (sections 2.13) and quantified as described (section 2.13.4).

### 5.3.7. Controls And Quantitation

Blank controls containing water only (0.5 ml) or water with d7-standards (50 µl) were included with each experiment. For quantitation of oxysterols and cholesterol, standard curves were constructed from samples prepared in water (0.5 ml) containing standards (10 µl each) representing a range of concentrations of cholesterol (0.5, 1, 5, 10, 20, 50, 100 and 500 µM) and oxysterols (5, 25, 50, 125, 250, 500 nM, 1 µM and 2 µM). Standards were processed as described for test samples (section 2.11.1). A new standard curve was generated during each assay to control for inter-assay differences in sample processing and extraction.

To calculate the accuracy and precision (inter-assay from n=6 replicates) of the analytical method (GC/MS) used in this chapter the following formulae were used:

- (1) Accuracy (% RME) = ((Mean – Reference value)/Reference value) x100
- (2) Precision (% CV) = (standard deviation/mean) x100

### 5.3.8. Statistical Analysis

All samples of blood (except from ApoE<sup>-/-</sup> mice) and aortae were obtained by pooling the tissue from two animals, as single aliquots, whereas microsomes and cytosols were analysed in duplicate. Statistical significance was determined using a non-parametric Mann-Whitney test for all data relating plasma/tissue analysis, and 1-way ANOVA for multiple comparisons, with a Tukey's posthoc test. In cases of normally distributed data, an unpaired Student's t-test was used for comparisons. Pearson correlations were performed to investigate associations between plasma concentrations of 7-oxysterols and those of cholesterol.

## 5.4. Results

### 5.4.1. Development Of A Method To Quantify The Levels Of 7-Oxysterols by GC/MS

An extensive literature search (summarized in Table 5 - 1) on use of mass spectrometry for detection of oxysterols in murine plasma and tissues was performed (Griffiths *et al.*, 2006; Murakami *et al.*, 2000; Volin, 2001). Notable parts of method development pertaining to each stage are given below. Minor adaptations of these methods are described with each type of biological sample used.

#### *a) Solvent extraction (Step A, Figure 5.4.3)*

Comparison of two different mixes of solvents used for initial extraction of oxysterols from plasma samples showed that these produced similar overall recovery of 7 $\beta$ OHC and 7-KC (Table 5 - 2). Centrifugation of samples was effective in breaking the emulsion between solvent mixture and plasma, and this was achieved more quickly with the diethylether:hexane mix (5 min, as opposed to 15 min with CHCl<sub>3</sub>:MeOH). Therefore for reasons of stability, subsequent lipid extractions from plasma samples were processed using the diethylether:hexane (2:3) solvent combination.

Table 5-1 Summary of published methods for detection of oxysterols in human and rat samples.

SAMPLE (ml)	Solvent		Solid Phase Extraction	Hydrolysis		2 <sup>nd</sup> Solvent Extraction	Detection Method	DERIVATISING METHOD		REFERENCES:
	Extraction			T (°C)	t (min)			Reagent	T (°C)	t (min)
LDL (1)	CHCl <sub>3</sub> :MeOH (2:1)		Diol, 300 mg	0°C	180	Diethyl ether	GC/MS	BSTFA	80	10 (Steffen <i>et al.</i> , 2006)
Plasma (0.5)	Diethylether:Hexane (2:3)		Diol, 300 mg	RT	120	NaCl, CHCl <sub>3</sub>	GC/MS	BSTFA	70	120 (Endo <i>et al.</i> , 2008)
Plasma (0.5)	2-Propanol:Hexane (3:7)		Si, 100 mg	RT	120	NaCl, CHCl <sub>3</sub>	GC/MS	3:2:1	60	30 (Larsson <i>et al.</i> , 2007)
Sera (0.2)	2-Propanol:Hexane (3:7)		Si, 100 mg	37°C	60	0.5% & 30%	GC/MS	4:1	80	45 (Prunet <i>et al.</i> , 2006)
CSF (0.5)	2-Propanol:Hexane (3:7)		Si, 100 mg	RT	120	CHCl <sub>3</sub>	GC/MS	3:2:1	60	30 (Leoni <i>et al.</i> , 2005)
Plasma (0.5)	CHCl <sub>3</sub> :MeOH (2:1)		Diol, 300 mg	4°C	180	Ethyl ether	GC/MS	DMF and BSTFA	80	10 (Siems <i>et al.</i> , 2005)
Plasma (0.5)	CHCl <sub>3</sub> :2-Propanol (1:1)		C18, 200 mg	50°C	120	n/p	HPLC	n/p	n/p	n/p (Burkard <i>et al.</i> , 2004)*
Rat Plasma (1)	NaCl (0.9%) in MeOH		Si, 100 mg	RT	120	0.5% & 30%	GC/MS	BSTFA	60	60 (Schweizer <i>et al.</i> , 2004)
Serum (0.2)	NaCl (0.1 g) in 3:2 mix		Glass wool	RT	180	Diethyl ether	GC/MS	BSTFA;1,4-Dioxan	70	30 (Alkazemi <i>et al.</i> , 2008)
Plasma (1)	CHCl <sub>3</sub> :MeOH (1:2)		Si, 100 mg	RT	120	CHCl <sub>3</sub>	GC/MS	3:2:1	60	30 (Zieden <i>et al.</i> , 1999)
Serum (0.05)	n/p		n/p	37°C	60	Hexane	LC/MS	Picolinyl ester	80	60 (Honda <i>et al.</i> , 2008) <sup>#</sup>

Abbreviations: GC/MS – Gas chromatography mass spectrometry; LC/MS – Liquid chromatography mass spectrometry; HPLC – High performance liquid chromatography; CSF – Cerebrospinal fluid; CHCl<sub>3</sub> – Chloroform; MeOH – Methanol; DMF – Dimethylformamide; NaCl – Sodium chloride; RT – Room temperature 22 °C; 3:2 (Hexane:2-Propanol, v/v); 0.35M potassium hydroxide (KOH) was used for hydrolysis apart from REF \* and <sup>#</sup> where 1M sodium hydroxide (NaOH) and 1N KOH (0.5 mL) were used; 3:2:1 was prepared from Pyridine:HMDS:TCMS (Pyridine:Hexamethyldisilazane:Trichloromethylsilazane, v/v/v); 4:1 was prepared from BSTFA:TCMS (Bis(trimethyl)silyltrifluoroacetamide:Trichloromethylsilazane, v/v); 0.5% mix (2-Propanol:Cyclohexane, v/v); 30% mix (2-Propanol:Cyclohexane, v/v); n/p, not performed.

**Table 5-2 Extraction of 7-oxysterols (7-KC and 7 $\beta$ OHC) from water and plasma.**

Percentage Recovery (%)			
		CHCl <sub>3</sub> : MeOH (2:1)	Diethylether : Hexane (2:3)
<b>7<math>\beta</math>OHC</b>	<b>H<sub>2</sub>O</b>	59	74
	<b>Plasma</b>	40	41
<b>7-KC</b>	<b>H<sub>2</sub>O</b>	100	84
	<b>Plasma</b>	68	61

Oxysterols were extracted into two different mixes of solvents (CHCl<sub>3</sub>:MeOH, 2:1 and diethylether:hexane, 2:3), in duplicate. 7-Oxysterols were quantified by GC/MS and recovery was calculated as a percentage of the peak area of standards following extraction compared to those without extraction. Data represent mean of duplicates, n=2.

*b) Solid phase extraction (SPE) (Step B, Figure 5.4.3)*

Following lipid extraction, the recovery of analytes was tested after elution from the diol columns. The highest achieved percentages of recovery were ~80 % (7-KC) and ~52 % (7 $\beta$ OHC).

*c) Hydrolysis (Saponification) (Step C, Figure 5.4.3)*

Tests were performed to check the effect of temperature on the recovery of 7 $\beta$ OHC and 7-KC following hydrolysis of plasma lipids (see Table 5 - 3). By performing cold hydrolysis, at 4°C, maximal recovery of both 7-KC and 7 $\beta$ OHC (and their deuterium-labelled isotopomers) was obtained (~100%), compared with warm (37°C) or RT-hydrolysis. Subsequently cold saponification was performed on ice using KOH (1 ml, 20% in methanol) and in the dark.



**Table 5-3 Percentage recovery of 7-oxysterols (7 $\beta$ OHC and 7-KC) upon hydrolysis.**

Step C	Hydrolysis					
	Cold		RT		Warm	
	7 $\beta$ OHC	7-KC	7 $\beta$ OHC	7-KC	7 $\beta$ OHC	7-KC
<b>Percentage Recovery (%)</b>	100	100	84	99	64	83

The recovery of 7-oxysterols (5  $\mu$ g) from water was tested following the procedure described in Figure 5.4 – 1. Hydrolysis (Step C) was performed at different temperatures (cold (4°C), RT (22 – 24°C) or Warm (37°C)) by incubating the samples with methanolic KOH (20 %, 0.5 ml, 2h). Analytes were detected by GC/MS and percentage recovery of 7-oxysterols from hydrolysed samples was calculated relevant to the peak areas of non-hydrolysed samples subjected to the same conditions (methanol, without KOH). The results show similar recoveries of oxysterols when hydrolysis is performed at cold or RT, compared to warm, although, greater efficiency is ensured if low temperature is maintained as low as possible during the hydrolysis. Data represent mean of duplicates, n=2.

*d) Solvent extraction (Step D, Figure 5.4.3)*

Following hydrolysis, 7 $\beta$ OHC and 7-KC were extracted into solvents; chloroform or diethyl ether (with 50  $\mu$ g/ml BHT). Use of either solvent resulted in reasonable and similar recoveries of 7 $\beta$ OHC and 7-KC (Table 5 - 4), but diethyl ether was ultimately used to achieve quicker phase separation.

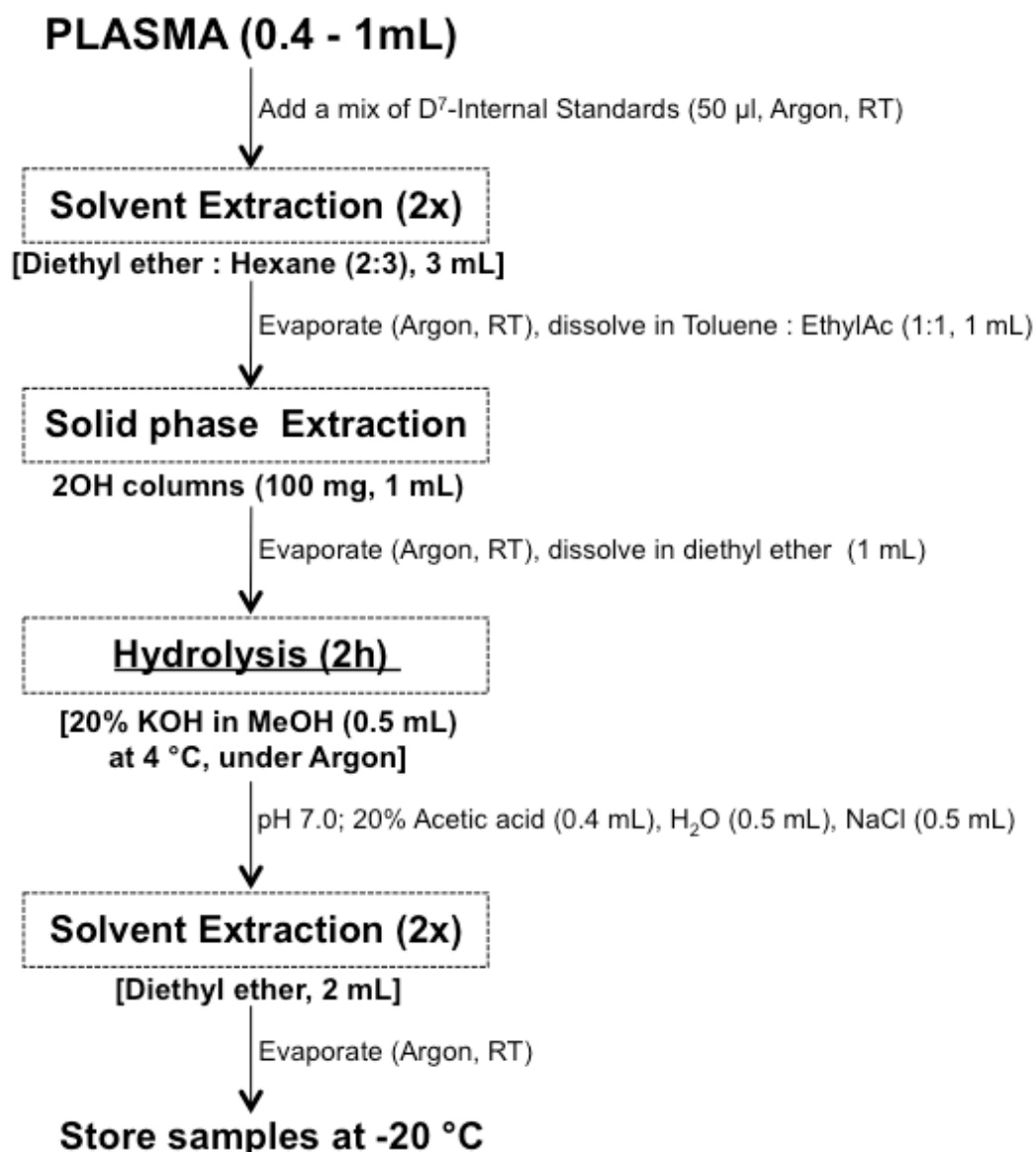
**Table 5-4 Solvent extraction of 7 $\beta$ OHC and 7-KC after hydrolysis.**

Step D	Chloroform		Diethyl ether	
	7 $\beta$ OHC	7-KC	7 $\beta$ OHC	7-KC
<b>Percentage Recovery (%)</b>	55	78	58	79

Samples were processed for extraction in steps outlined in Figure 5.4 – 1. Efficiency of extraction (Step D) was compared using two solvents, chloroform (CHCl<sub>3</sub>) or diethyl ether. The percentage recovery was calculated by expressing the peak areas as a ratio to those of non-extracted samples. The results show similar percentage recovery with either solvent. However diethyl ether was selected as it allowed a quicker phase separation. Data represent mean of duplicates, n=2.

#### **5.4.1.1. Detection Of Oxysterols In Biological Fluids And Tissue**

Based on the method development, a final protocol was adopted and used for subsequent analyses and detection of oxysterols in murine biological fluids and tissues. The full method is outlined in Figure 5.4 – 1.



**Figure 5.4-1 Optimized method for the extraction of oxysterols from biological fluids and tissues.**

Detection of oxysterols in samples (0.4 - 1ml) of murine biological fluids and tissues includes Step A - Solvent extraction; Step B - Solid phase extraction, SPE; Step C – Hydrolysis; Step D - Solvent extraction. Subsequent detection of oxysterols was performed by GC/MS (section 2.13.3) with the ions selected for analysis as listed in Table 2 - 1. Toluene : EthylAc (1:1), Toluene:Ethylacetate; KOH, Potassium hydroxide; MeOH, Methanol; NaCl, Sodium chloride; RT, Room temperature.

#### 5.4.1.2. **Precision And Accuracy Of The Method Developed For Detection Of Oxysterols**

The reproducibility of the preferred assay was investigated by analyzing the same biological fluids and tissues from mice enriched with known amounts of oxysterols (0.5-50 ng) or cholesterol (0.19 – 20 µg) in a 0.2 ml aliquot of sample. To account for endogenous concentrations of these compounds, baseline levels were always subtracted from those in the enriched samples. Standard curves with known amounts of 7KC, 7βOHC, 7αOHC or cholesterol were prepared and the linearity of the response assessed and reported in Table 5 - 5 as the correlation coefficient (r). The inter-assay coefficient of variation for precision was ± 12 %, and the RME of accuracy: 3.8 – 15%.

**Table 5-5 Calibration curves for 7-oxysterols and cholesterol**

Oxysterols	Range	(n)	Linear regression equation	Correlation coefficient (r)
7αOHC	1.0 – 20 ng	4	$Y = 0.0006X + 0.0043$	0.978
7βOHC	0.5 – 50 ng	6	$Y = 0.0023X - 0.0254$	0.999
7-KC	0.9 – 50 ng	6	$Y = 0.0023X - 0.0255$	0.986
Cholesterol	0.19 – 20 µg	5	$Y = 0.0922X + 0.0226$	0.987

Calibration curves were constructed for the amount of each 7-oxysterol (X) against the corresponding ratio of peak-area ratio (Y) of derivatised oxysterol(s) to that of a derivatised-deuterated internal standard. For detection of 7α-hydroxycholesterol (7αOHC), [<sup>2</sup>H<sub>7</sub>]-7β-hydroxycholesterol (7βOHC) was used as an internal standard. Linearities were checked by linear regression, and the equation for the line of best fit given as in the table. Correlation coefficients of  $r > 0.97$  were achieved. 7-KC, 7-ketocholesterol.

## 5.4.2. Quantitation Of Plasma Lipids In Experimental Animals

### 5.4.2.1. *Lipid Levels In Murine Plasma – Method Development*

Cholesterol and oxysterols were detected in plasma from transgenic 5 $\alpha$ SRD1<sup>+/-</sup> male and female mice, which were available for use, in order to verify the utility of the optimized extraction method and refine the calibration range. The nature of this genetic background will not be discussed further, however, phenotypically their lipid profiles are not different from those of control (C57Bl/6) mice (unpublished, Livingstone *et al.*). Consistent levels of cholesterol ( $\mu$ g/ml) and 7-oxysterols (ng/ml) were determined in all samples tested (Table 5 - 6). Levels of cholesterol were higher than those of 7-oxysterols by three orders of magnitude. The order of abundance of 7-oxysterols in murine plasma was 7-KC > 7 $\alpha$ OHC > 7 $\beta$ OHC; all present at ng/ml concentrations.

In both genders plasma 7-KC levels were higher than those of 7 $\beta$ OHC. The levels of 7-KC and the ratio of 7-KC to 7 $\beta$ OHC (7-KC/7 $\beta$ OHC) were higher in females compared with male mice. In addition, when levels of 7-KC and 7 $\beta$ OHC were related to the total plasma cholesterol, both ratios of 7-KC/Chol and 7 $\beta$ OHC/Chol were significantly higher in females compared with male mice.

**Table 5-6 Comparison of lipid levels in plasma from male and female 5 $\alpha$ SRD1<sup>+/-</sup> mice for method development.**

Plasma	Male	Female	p-value
<b>Cholesterol (<math>\mu</math>g/ml)</b>	66.5 $\pm$ 3.6 (172 $\mu$ M)	42.5 $\pm$ 3.5 (109.6 $\mu$ M)	<b>0.001</b>
<b>7-Oxysterols (ng/ml)</b>			
<b>7<math>\alpha</math>OHC</b>	38.8 $\pm$ 8.2 (0.097 $\mu$ M)	21.3 $\pm$ 1.4 (0.053 $\mu$ M)	0.062
<b>7<math>\beta</math>OHC</b>	21.6 $\pm$ 1.4 (0.054 $\mu$ M)	28.2 $\pm$ 1.9 (0.071 $\mu$ M)	0.074
<b>7-KC</b>	34.5 $\pm$ 8.8 (0.086 $\mu$ M)	64.8 $\pm$ 10.6 (0.162 $\mu$ M)	<b>0.026</b>
<b>Ratios</b>			
<b>7-KC/7<math>\beta</math>OHC</b>	1.527 $\pm$ 0.278	2.221 $\pm$ 0.205	<b>0.050</b>
<b>7-KC/Chol x100</b>	0.055 $\pm$ 0.017	0.158 $\pm$ 0.029	<b>0.012</b>
<b>7-<math>\beta</math>OHC/Chol x100</b>	0.034 $\pm$ 0.004	0.069 $\pm$ 0.098	<b>0.007</b>

Mean plasma cholesterol and 7-oxysterol concentrations were compared between male and female 5 $\alpha$ Reductase1<sup>+/-</sup> mice (5 $\alpha$ SRD1<sup>+/-</sup>). Cholesterol levels were higher in males than in females. Levels of 7-ketocholesterol (7-KC) and all plasma ratios were significantly higher in female than in male mice. In addition, levels of 7-KC appeared higher than 7 $\beta$ -hydroxycholesterol (7 $\beta$ OHC). 7 $\alpha$ OHC, 7 $\alpha$ -hydroxy-cholesterol. All data are mean  $\pm$  SEM, compared by non-parametric Mann-Whitney test (levels), unpaired Student t-test (ratios), n=6, p<0.05, p<0.01.

#### 5.4.2.2. ***Does Disruption Of 11 $\beta$ HSD1 In Mice Alter The Levels Of Plasma 7-Oxysterols?***

Plasma concentrations of 7-oxysterols and cholesterol were measured in C57Bl/6 (control) and 11 $\beta$ HSD1<sup>-/-</sup> mice. Cholesterol levels in control mice were higher than in the previous genotype (5 $\alpha$ SRD1<sup>+/-</sup>), (see Tables 5 - 6 and 5 - 7), despite having similar body weights (data not shown). Cholesterol levels were significantly lower in the 11 $\beta$ HSD1<sup>-/-</sup> than in control mice (Table 5 - 7).

In both control and 11 $\beta$ HSD1<sup>-/-</sup> mice, 7-oxysterols were present in the range of 5-60 ng/ml. Levels of 7-KC in the control mice were higher than those of 7 $\beta$ OHC, in agreement with the findings from pilot work (Section 5.4.2). In mice lacking 11 $\beta$ HSD1 activity, there was a trend for increased concentrations of 7-KC (p=0.08) compared with C57Bl/6 (see Table 5 - 7), but the levels of 7 $\alpha$ OHC or 7 $\beta$ OHC were similar in these strains. Accordingly, genetic disruption of 11 $\beta$ HSD1 resulted in a significant increase in the ratios of 7-KC/7 $\beta$ OHC compared with controls.

In C57Bl/6 normolipidemic mice, total circulating 7-KC, 7 $\beta$ OHC and 7 $\alpha$ OHC levels were 10,000 times lower than total cholesterol. The ratio of 7-KC to total cholesterol (7-KC/Chol) was significantly higher in the plasma of 11 $\beta$ HSD1<sup>-/-</sup> mice compared with control, as was the ratio of 7 $\beta$ OHC/Chol (Table 5 - 7). When levels of 7-oxysterol were correlated with the total cholesterol levels, a positive association was observed in control plasma samples for 7 $\beta$ OHC (p=0.026 r=0.814, n=8) and 7-KC (p=0.029 r=0.806; n=7) but not for (7 $\alpha$ OHC p=0.39 r=0.38, n=8). In the plasma samples from 11 $\beta$ HSD1<sup>-/-</sup> mice there a significant correlation was not observed between the concentrations of any of the 7-oxysterols detected and total cholesterol levels (7 $\alpha$ OHC p=0.95 r= -0.02, n=8; 7 $\beta$ OHC p=0.51 r=0.27, n=8; 7-KC, p=0.31 r=0.45, n=7).

**Table 5-7 Disruption of 11 $\beta$ HSD1 in mice alters plasma levels of cholesterol and 7-oxysterols.**

Plasma	C57Bl/6	11 $\beta$ HSD1 <sup>-/-</sup>	p-value
<b>Cholesterol (<math>\mu</math>g/ml)</b>	398.4 $\pm$ 74.4 (1.03 mM)	174.6 $\pm$ 54.6 (0.451 $\mu$ M)	<b>0.02</b>
<b>7-Oxysterols (ng/ml)</b>			
<b>7<math>\alpha</math>OHC</b>	22.9 $\pm$ 2.8 (0.057 $\mu$ M)	20.5 $\pm$ 1.6 (0.051 $\mu$ M)	0.89
<b>7<math>\beta</math>OHC</b>	9.1 $\pm$ 2.3 (0.023 $\mu$ M)	9.5 $\pm$ 0.9 (0.024 $\mu$ M)	0.72
<b>7-KC</b>	36.5 $\pm$ 8.9 (0.091 $\mu$ M)	53.5 $\pm$ 6.7 (0.133 $\mu$ M)	0.08
<b>Ratios</b>			
<b>7-KC/7<math>\beta</math>OHC</b>	4.142 $\pm$ 0.373	5.445 $\pm$ 0.471	<b>0.04</b>
<b>7-KC/Chol x100</b>	0.014 $\pm$ 0.005	0.039 $\pm$ 0.009	<b>0.01</b>
<b>7<math>\beta</math>OHC/Chol x100</b>	0.003 $\pm$ 0.001	0.007 $\pm$ 0.002	<b>0.03</b>

Levels of cholesterol ( $\mu$ g/ml) and 7-oxysterols (ng/ml) were determined in plasma from 11 $\beta$ HSD1<sup>-/-</sup> and control C57Bl/6 mice. Cholesterol levels were reduced upon disruption of 11 $\beta$ HSD1. In both genotypes 7-oxysterols were present in the range of 5-60 ng/ml. Levels of 7-ketocholesterol (7-KC) were higher than 7 $\beta$ -hydroxycholesterol (7 $\beta$ OHC) or 7 $\alpha$ -hydroxycholesterol (7 $\alpha$ OHC). A trend was revealed for increased 7-KC levels, with an associated increase in the ratio of 7-KC/7 $\beta$ OHC, upon disruption of 11 $\beta$ HSD1. Similarly, the ratios of 7-oxysterols to the total cholesterol were significantly increased in plasma of 11 $\beta$ HSD1<sup>-/-</sup> mice. All data are mean  $\pm$  SEM, compared by non-parametric Mann-Whitney test (levels) or unpaired Student t-test (ratios), n=8, p<0.05.



### 5.4.3. Quantitation Of Tissue Lipid In Experimental Animals

#### 5.4.3.1. *Does Disruption Of 11 $\beta$ HSD1 Alter The Levels Of 7-Oxysterols In Hepatic Fractions In Mice?*

7 $\beta$ OHC and 7-KC were readily detected in hepatic sub-cellular fractions from C57Bl/6 and 11 $\beta$ HSD1<sup>-/-</sup> mice (Table 5 - 8). In livers from C57Bl/6 mice, both 7-oxysterols were equally abundant in the microsomal and cytosolic fractions. As in plasma, the levels of 7-KC in the microsomal preparations, were significantly higher than those of 7 $\beta$ OHC ( $p=0.01$ ), although this was not the case in the cytosolic fraction in which 7 $\beta$ OHC and 7-KC were present with similar abundance. Disruption of 11 $\beta$ HSD1 resulted in significantly lower levels of both 7 $\beta$ OHC and 7-KC levels inside the microsomes compared with control. The ratios of 7-KC/7 $\beta$ OHC were significantly reduced in 11 $\beta$ HSD1<sup>-/-</sup> mice compared with C57Bl/6. The levels of 7-oxysterols in the cytosols of 11 $\beta$ HSD1<sup>-/-</sup> mice were almost undetectable (Limit of detection L.O.D. = 0.01 – 0.03 ng/mg).

#### 5.4.4. What Are The Plasma Levels Of 7-Oxysterols In Atherosclerotic ApoE<sup>-/-</sup> Mice?

Plasma concentrations of 7-oxysterols and cholesterol were measured in ApoE<sup>-/-</sup> mice (Table 5 - 9). Cholesterol levels were higher than in the previous genotypes (5 $\alpha$ SRD1<sup>+/-</sup> and 11 $\beta$ HSD1<sup>-/-</sup>), (see Tables 5 - 6 & 5 - 8). 7-Oxysterols were present in the range of 120 – 560 ng/ml. Levels of 7-KC were higher than those of 7 $\beta$ OHC, in agreement with previous findings and as seen by comparison of the ratios of 7-KC/7 $\beta$ OHC. Total circulating 7-KC, 7 $\beta$ OHC and 7 $\alpha$ OHC levels were 10,000 times lower than total cholesterol. The ratios of 7-KC/Chol were higher than those of 7 $\beta$ OHC/Chol.

**Table 5-8 Disruption of 11 $\beta$ HSD1 in mice alters the levels of 7-oxysterols in hepatic fractions.**

<b>7-Oxysterols (ng/mg protein)</b>	<b>C57Bl/6</b>	<b>11<math>\beta</math>HSD1<sup>-/-</sup></b>	<b>p-value</b>
<b>Microsomes</b>			
<b>7<math>\beta</math>OHC</b>	16.1 $\pm$ 11.4	7.8 $\pm$ 0.7	<b>0.03</b>
<b>7-KC</b>	51.3 $\pm$ 28.3	3.0 $\pm$ 1.1	<b>0.01</b>
<b>7-KC/7<math>\beta</math>OHC</b>	1.9 $\pm$ 0.1	0.3 $\pm$ 0.1	<b>0.001</b>
<b>Cytosols</b>			
<b>7<math>\beta</math>OHC</b>	25.3 $\pm$ 13.4	0.03 $\pm$ 0.01	0.40
<b>7-KC</b>	39.8 $\pm$ 19.2	0.02 $\pm$ 0.01	0.27
<b>7-KC/7<math>\beta</math>OHC</b>	2.5 $\pm$ 0.7	n/d	n/c

The levels of 7-ketocholesterol (7-KC) and 7 $\beta$ -hydroxycholesterol (7 $\beta$ OHC) were quantified in hepatic microsomal (100  $\mu$ l) and cytosolic (200  $\mu$ l) fractions from 11 $\beta$ HSD1<sup>-/-</sup> and C57Bl/6 mice. Levels of 7-KC were higher than those of 7 $\beta$ OHC in microsomes from control mice ( $p < 0.01$ ), and this difference was reversed in microsomes from 11 $\beta$ HSD1<sup>-/-</sup> mice. Disruption of 11 $\beta$ HSD1 caused significant reduction in microsomal levels of 7-KC and 7 $\beta$ OHC. 7-KC and 7 $\beta$ OHC were present at a similar level in the hepatic cytosolic fraction of C57Bl/6 mice. Levels of both 7-oxysterols in hepatic cytosols from 11 $\beta$ HSD1<sup>-/-</sup> mice were 0.02-0.04 ng/mg (LOD 0-0.03 ng/mg), and detected in only 3 out of 8 samples. Peaks for 7 $\alpha$ -hydroxycholesterol were not quantified. All data are corrected for protein concentration (~20 mg/ml) and represent mean  $\pm$  SEM, compared by a non-parametric Mann-Whitney test,  $n=8$ ,  $p < 0.05$ ,  $p < 0.01$ . n/d, not detected; n/c, not calculated.

**Table 5-9 Plasma levels of cholesterol and 7-oxysterols in atherosclerotic ApoE<sup>-/-</sup> mice.**

Plasma	ApoE <sup>-/-</sup>
<b>Cholesterol (mg/ml)</b>	<b>4.4 ± 0.4</b> <b>(12.0 mM)</b>
<b>7-Oxysterols (ng/ml)</b>	
<b>7αOHC</b>	171.7 ± 42.1 (0.427 μM)
<b>7βOHC</b>	163.2 ± 15.5 (0.406 μM)
<b>7-KC</b>	493.6 ± 88.7 (1.227 μM)
<b>Ratios</b>	
<b>7-KC/7βOHC</b>	3.112 ± 0.651
<b>7-KC/Chol x100</b>	0.012 ± 0.003
<b>7βOHC/Chol x100</b>	0.004 ± 0.001

The levels of 7-ketocholesterol (7-KC) and 7β-hydroxycholesterol (7βOHC) were quantified in plasma (250-300 μl) from ApoE<sup>-/-</sup> mice fed chow diet. Levels of 7-KC were higher than 7βOHC or 7α-hydroxycholesterol (7αOHC). Levels of 7-KC and 7βOHC in plasma of ApoE<sup>-/-</sup> mice were higher compared to those detected in plasma of C57Bl/6 mice. All data represent mean ± SEM, n=6.

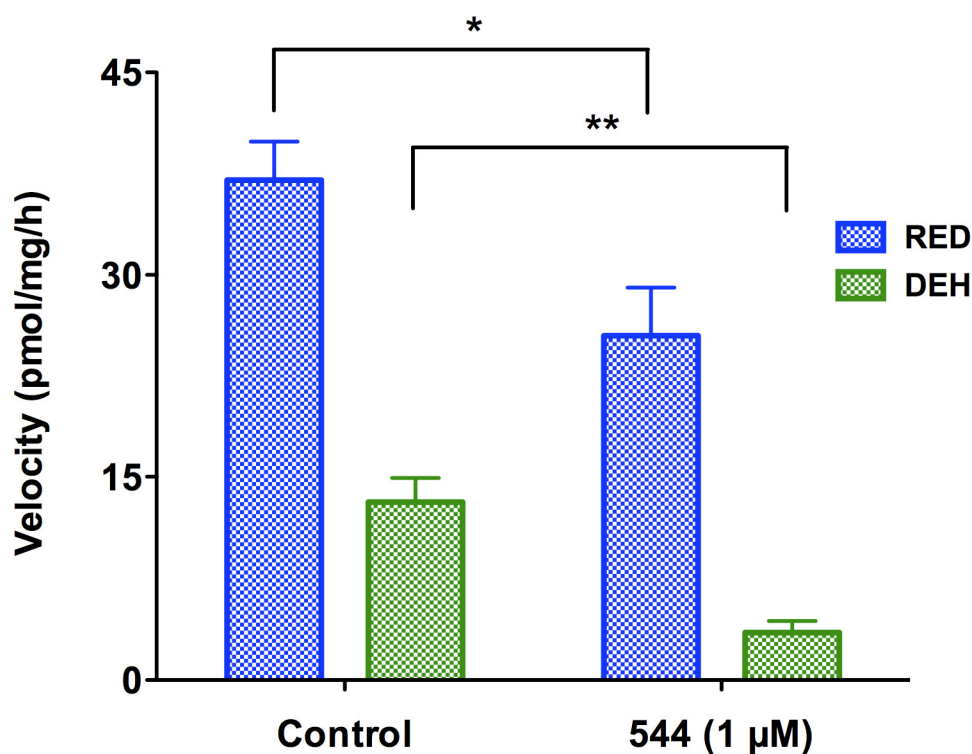
#### 5.4.5. Does Inhibition Of 11 $\beta$ HSD1 Influence The Levels Of 7-Oxysterols in ApoE<sup>-/-</sup> Mice?

##### 5.4.5.1. Determination Of 11 $\beta$ HSD1 Inhibition In Vitro

The ability of compound 544 (11 $\beta$ HSD1 inhibitor) to inhibit 11 $\beta$ HSD1 activity was tested *in vitro*. Measurements of the reaction rate of glucocorticoid inter-conversion by 11 $\beta$ HSD (reductase and dehydrogenase) were assessed in the presence of vehicle or compound 544 (1  $\mu$ M). In the presence of inhibitor reaction velocities of reduction of 11-dehydrocorticosterone were lower than in controls (Figure 5.4 – 2).

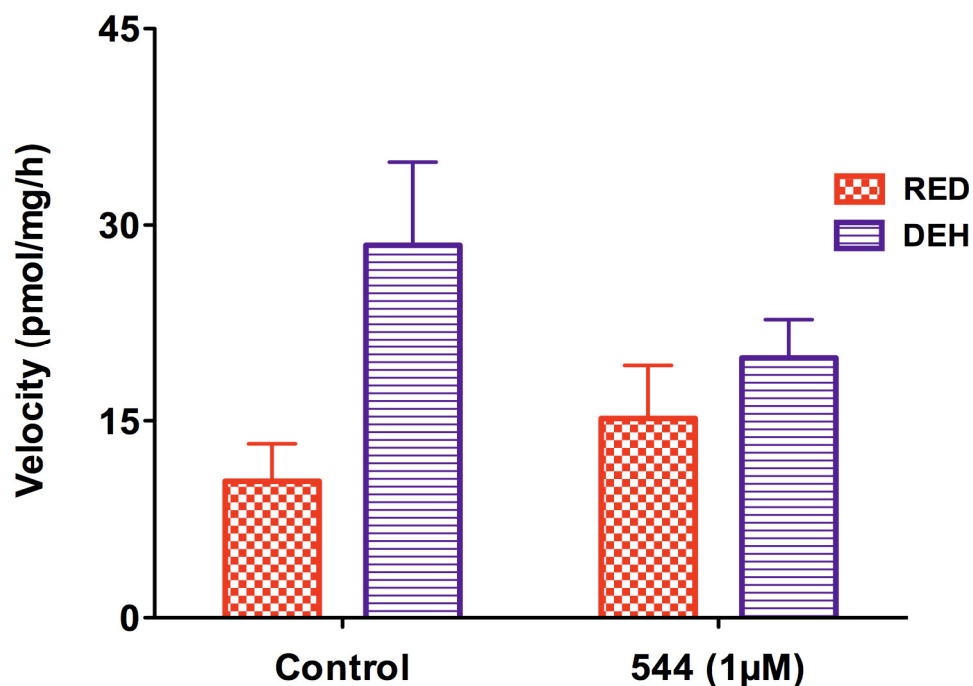
In addition, the ability of compound 544 to inhibit the metabolism of 7-oxysterols by 11 $\beta$ HSD1 was tested *in vitro*. The reaction rates of conversion of 7-KC to 7 $\beta$ OHC (reductase) as well as those of 7 $\beta$ OHC to 7-KC (dehydrogenase) were assessed in the presence of vehicle or compound 544 (1  $\mu$ M). In the control cells both reactions proceeded (as reported in Chapter 3, Figure 3.4 – 3) and dehydrogenation of 7 $\beta$ OHC occurred at a faster rate. Addition of compound 544 did not inhibit dehydrogenation of 7 $\beta$ OHC to 7-KC nor reduction of 7-KC to 7 $\beta$ OHC (Figure 5.4 – 3).

The amounts of 7-oxysterols distributed between the medium and cells was calculated as a percentage of total amount of oxysterols recovered (7-KC+7 $\beta$ OHC) from both compartments, and are shown in Figure 5.4 – 4. On average total of 70 - 82 % of oxysterols were recovered. In the assay of *reduction*, 7-KC (expressed as a percentage of total oxysterol recovered) remained mostly in the medium (> 40%) and about half this amount was detected in the cells (<20%). In the same assay, 7 $\beta$ OHC was generated and detected in cells (~5%) and in the medium (~12%). Upon measuring the reductase activity in the presence of compound 544, there was no obvious change in proportions of distributed 7-oxysterols compared to control.



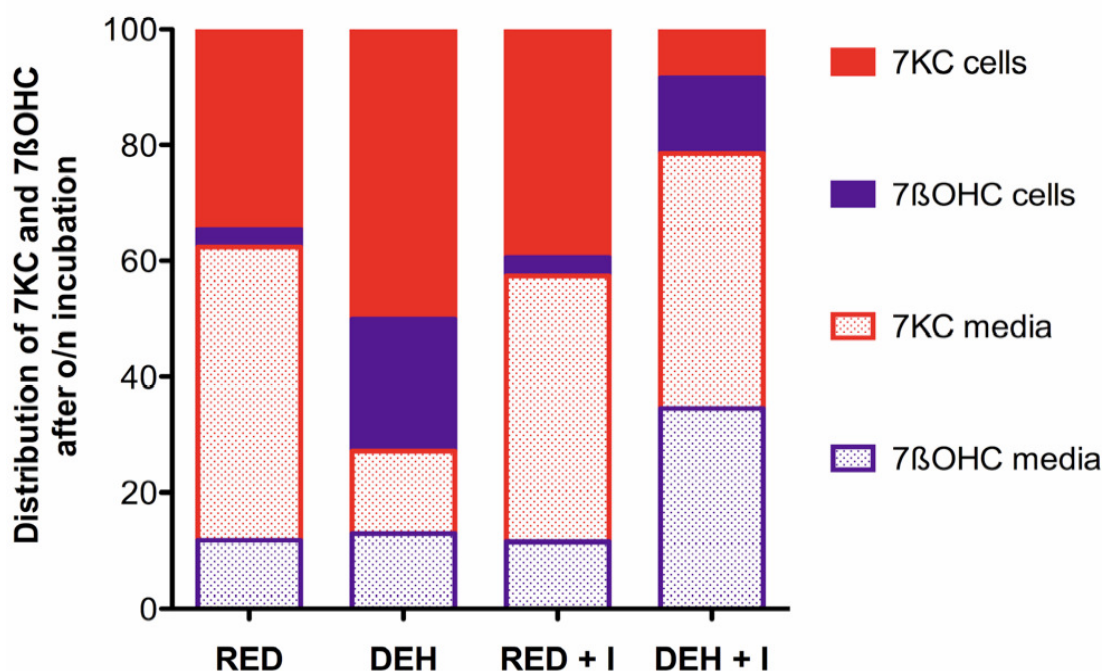
**Figure 5.4-2 Selective inhibition of 11βHSD1 *in vitro* reduces both reduction and dehydrogenation of glucocorticoids.**

HEK293 cells stably-transfected to generate murine 11βHSD1 recombinant enzyme were used to assess inhibition of reduction of [ $^3\text{H}$ ]-11-dehydrocorticosterone (green bars) and inhibition of dehydrogenation (blue bars) of [ $^3\text{H}$ ] $_4$ -corticosterone. Substrate (30 nM in DMEM) was added to the cells to assess the reaction rates in the presence of vehicle (5% w/v 2-hydroxypropyl-β-cyclodextrin), or 11βHSD1 inhibitor (1 μM, compound 544 in 5% w/v 2-hydroxypropyl-β-cyclodextrin, 3h). The data show that compound 544 non-selectively inhibits the activity of 11βHSD1, in both reduction and dehydrogenation of glucocorticoids. All data represent mean  $\pm$  SEM compared by an unpaired Student's t-test, n=6, \*p<0.05, \*\*p<0.01.



**Figure 5.4-3 Inhibition of 7-oxysterol metabolism by 11 $\beta$ HSD1 *in vitro*.**

HEK293 cells stably transfected to generate murine recombinant 11 $\beta$ HSD1 were used to assess inter-conversion of alternative substrates. The velocities of reduction of 7-ketocholesterol (7-KC, red bars) and dehydrogenation of 7 $\beta$ -hydroxycholesterol (7 $\beta$ OHC, purple bars) by m11 $\beta$ HSD1 were assessed. Substrates (1  $\mu$ M in DMEM) were added to the cells and reaction rates were assessed in the presence of vehicle control (5% w/v, 2-hydroxypropyl- $\beta$ -cyclodextrin), or 11 $\beta$ HSD1 inhibitor (Compound 544, 1  $\mu$ M, 16h). The data show that compound 544 does not inhibit the 11 $\beta$ HSD1-mediated reduction of 7-KC or dehydrogenation of 7 $\beta$ OHC. All data represent mean  $\pm$  SEM; compared using unpaired Student's t-test, n=6.



**Figure 5.4-4 Substrate distribution between the cells and medium upon inhibition of 11βHSD1-mediated 7-oxysterol conversion *in vitro*.**

The distribution of substrates (7-ketocholesterol (7-KC) and 7β-hydroxycholesterol (7βOHC); 1 μM) across the cellular and extracellular compartments was expressed as a percentage (%) of a total amount of oxysterols recovered. Reactions were performed in both reductase and dehydrogenase directions in the absence (vehicle) and in the presence of inhibitor (I, compound 544, 1 μM). For each reaction, % formed product or % remaining substrate was determined: 7-KC in the cell (full red), 7βOHC in the cell (full purple), 7-KC in the medium (pale red), 7βOHC in the medium (pale purple). On average total of 70 - 82 % of oxysterols was recovered. The addition of inhibitor in the reduction direction did not change the proportions of distributed 7-oxysterols compared to control. A noticeable shift in proportions of distributed 7-oxysterols was seen with inhibitor in the dehydrogenation direction compared to control. The inhibitor facilitated export of 7-oxysterols from cells and majority was recovered from the medium.

During the assay of *dehydrogenation*, the majority (>50%) of 7 $\beta$ OHC (expressed as a percentage of total oxysterol recovered) was converted to 7-KC and between 15-20% of 7 $\beta$ OHC was left in the medium and cells. Most of 7-KC that was formed remained in the cells (~50%) although some (~12%) was found in the medium. Upon measuring the dehydrogenase activity in the presence of compound 544, there seem to be a noticeable shift in proportions of distributed 7-oxysterols, such that 7-oxysterols were exported from cells and majority was recovered from the medium.

#### **5.4.5.2. Determination Of Lipid Levels In ApoE<sup>-/-</sup> Mice Following Inhibition Of 11 $\beta$ HSD1**

7-Oxysterols and cholesterol were quantified in the hepatic fractions from ApoE<sup>-/-</sup> mice. Lipid levels in hepatic microsomes (Table 5 - 10) and cytosols (Table 5 - 11) from ApoE<sup>-/-</sup> mice treated with an inhibitor of 11 $\beta$ HSD1 (compound 544) and matched control (5%, 2-hydroxypropyl- $\beta$ -cyclodextrin) are given in Tables 5 - 10 and 5 - 11, respectively. The levels of hepatic lipids in the ApoE<sup>-/-</sup> mice were between 5 to 9 times higher compared to those determined in the control mice, reported in Table 5 - 8.

Levels of cholesterol or 7-KC and 7 $\beta$ OHC in hepatic microsomal fractions were not different between control and 11HSD1 inhibitor-treated ApoE<sup>-/-</sup> groups. The ratio of 7-KC/7 $\beta$ OHC also remained unchanged between the two groups. Normalisation of 7-oxysterols to the total cholesterol levels in the microsomes, did not reveal difference in either the ratio of 7-KC to total cholesterol (7-KC/Chol) or 7 $\beta$ OHC/Chol (see Table 5 - 10).

Cholesterol levels in the hepatic cytosolic fractions were present in the similar range (150 – 250  $\mu$ g/mg) to those in hepatic microsomal fractions from ApoE<sup>-/-</sup> mice (Tables 5 - 10 and 5 - 11). The levels of 7-KC and 7 $\beta$ OHC in control cytosolic fractions were similar, and did not change upon treatment of ApoE<sup>-/-</sup> mice with 11 $\beta$ HSD1 inhibitor.



The ratios of 7KC/7 $\beta$ OHC in the hepatic cytosols of ApoE<sup>-/-</sup> control mice (Table 5 - 11) were not different ( $p>0.1$ ) from those in the hepatic microsomes of the same animals (see Table 5 - 10). Normalisation of 7-oxysterols to the total cholesterol levels in the cytosols did not reveal differences in either the ratio of 7-KC/Chol or 7 $\beta$ OHC/Chol (see Table 5 - 11).

**Table 5-10 Lipid levels in hepatic microsomes of ApoE<sup>-/-</sup> mice.**

Microsomes	ApoE <sup>-/-</sup> (Vehicle)	ApoE <sup>-/-</sup> (Inhibitor)	p-value
<b>Cholesterol (<math>\mu</math>g/mg)</b>	242.5 $\pm$ 68.5	149.9 $\pm$ 44.4	0.28
<b>7-Oxysterols (ng/mg protein)</b>			
<b>7<math>\beta</math>OHC</b>	137.8 $\pm$ 73.5	85.6 $\pm$ 12.2	0.49
<b>7-KC</b>	185.5 $\pm$ 77.8	106.6 $\pm$ 21.3	0.35
<b>Ratios</b>			
<b>7-KC/7<math>\beta</math>OHC</b>	1.67 $\pm$ 0.22	1.39 $\pm$ 0.27	0.44
<b>7-KC/Chol x100</b>	0.85 $\pm$ 0.26	1.44 $\pm$ 0.74	0.69
<b>7-<math>\beta</math>OHC/Chol x100</b>	0.59 $\pm$ 0.24	0.99 $\pm$ 0.45	0.45

Levels of 7-oxysterols (ng/mg) and cholesterol ( $\mu$ g/mg) were not different in hepatic microsomes from ApoE<sup>-/-</sup> mice treated with an 11 $\beta$ HSD1 inhibitor (compound 544, 10 mg/kg/day, in food, 8 weeks) and matched ApoE<sup>-/-</sup> mice treated with vehicle (5% w/v, 2-hydroxypropyl- $\beta$ -cyclodextrin in food). The ratios of 7-ketocholesterol (7-KC)/7 $\beta$ -hydroxycholesterol (7 $\beta$ OHC) as well as the ratio of each 7-oxysterol to cholesterol were also unchanged. Data are corrected for protein concentration and represent mean  $\pm$  SEM, all compared by unpaired Student's t-test,  $n=6$ ,  $p>0.05$ .

**Table 5-11 Lipid levels in hepatic cytosols of ApoE<sup>-/-</sup> mice.**

Cytosols	ApoE <sup>-/-</sup> (Vehicle)	ApoE <sup>-/-</sup> (Inhibitor)	p-value
Cholesterol (µg/mg)	272.1 ± 76.9	262.8 ± 67.7	0.92
<b>7-Oxysterols (ng/mg protein)</b>			
7βOHC	154.8 ± 39.0	102.6 ± 19.5	0.25
7-KC	285.5 ± 117	160.7 ± 27.0	0.32
<b>Ratios</b>			
7-KC/7βOHC	1.60 ± 0.19	1.62 ± 0.09	0.94
7-KC/Chol x100	1.66 ± 0.89	0.89 ± 0.24	0.42
7-βOHC/Chol x100	0.87 ± 0.34	0.53 ± 0.11	0.35

Levels of 7-oxysterols (ng/ml) and cholesterol (µg/ml) were not different in hepatic cytosols from ApoE<sup>-/-</sup> mice treated with an 11βHSD1 inhibitor (compound 544, 10 mg/kg/day, in food, 8 weeks) and matched ApoE<sup>-/-</sup> mice treated with vehicle (5% w/v, 2-hydroxypropyl-β-cyclodextrin in food). The ratios of 7-ketocholesterol (7-KC)/7β-hydroxycholesterol (7βOHC) as well as the ratio of each 7-oxysterol to cholesterol were also unchanged. All data are corrected for protein concentration and represent mean ± SEM, all compared by unpaired Student's t-test, n=6, p>0.05.

#### 5.4.5.3. Levels Of 7-Oxysterols In Aortae Of C57Bl/6 Mice

Attempts were made to determine the levels of 7-oxysterols in whole aortae, assuming that some of the circulating 7-oxysterols may be sequestered by cells in the vessel wall. For this purpose intact aortae from C57Bl/6 mice were used in which levels of 7-oxysterols were found to be close to the detection limit (4-5 times background). Levels were measured after pooling two aortae (n=5-6), and higher levels (p=0.05) of 7-KC (1.41 ± 0.81 ng/mg tissue) were detected compared to those of 7βOHC (0.16 ± 0.06 ng/mg tissue). The ratio of 7-KC/7βOHC (12.69 ± 3.40) was higher in the aortic samples (p<0.01, 1-way ANOVA, Tukey's post hoc test) compared to that in plasma (p<0.01) (Table 5 - 7) or hepatic subcellular fractions (p<0.01) of C57Bl/6 mice (Tables 5 - 8).

## 5.5. Discussion

The work presented in this chapter tested the hypothesis that 7-KC would accumulate in the plasma and tissues upon inhibition of 11 $\beta$ HSD1. The initial aim of this work was to develop a sufficiently sensitive method (i.e. GC/MS) to measure circulatory levels of 7-oxysterols and cholesterol in the mouse. In brief, the data in this chapter show that levels of 7-oxysterols in plasma of chow-fed C57Bl/6 mice, are in a range between 10-100 ng/ml (0.03-0.25  $\mu$ M); similar to the levels reported in human plasma or other murine models previously studied (for references see Table 5 - 1). Hepatic sterols were equally distributed between microsomes and cytosol, whereas very low amounts of oxysterols were detected in healthy aortae. Consistently, the levels of 7-KC were higher than those of 7 $\beta$ OHC in all model systems studied. Genetic disruption of 11 $\beta$ HSD1 caused a lowering of total circulating cholesterol, but produced only subtle changes in the plasma levels of 7-oxysterols. In the liver of 11 $\beta$ HSD1<sup>-/-</sup> mice, both 7 $\beta$ OHC and 7-KC were substantially reduced compared to C57Bl/6 mice. An 11 $\beta$ HSD1 inhibitor (compound 544) inhibited metabolism of glucocorticoids but not 7-oxysterols by this enzyme, and did not alter tissue levels of 7-oxysterols. This important finding reflects on the specificity of compound 544 for 11 $\beta$ HSD1-mediated metabolism of glucocorticoids, indicating that inhibition of this pathway accounts for the main effects induced by this compound. These findings are important for improving the understanding of therapeutic benefits of 11 $\beta$ HSD1 inhibition in diseases such as atherosclerosis and hyperlipidaemia (Hermanowski-Vosatka *et al.*, 2005).

### 5.5.1. Method Development - Detection Of Oxysterols In Murine Biological Fluids And Tissues

The initial aim for this chapter was to develop a method that allowed efficient recovery of oxysterols from murine plasma. The detection of oxysterols in various murine tissues, cells and fluids from experimental animals required a sensitive, reproducible and accurate analytical method with minimal interference from other peaks in the mass chromatographic trace. Early investigations in the field of

oxysterols were severely hampered by the lack of such an analytical method and, as a consequence, studies often arrived at vastly different conclusions (Brown and Jessup, 1999). Although there are many GC/MS-based methods for detection of oxysterols levels in plasma and tissues (see Table 5 - 1), several research groups have reported difficulties associated with stability during sample preparation (Dzeletovic et al., 1995a; Kritharides et al., 1993). For the findings presented in this chapter, it was important to adapt a single method from the literature for determination of low concentrations of 7-oxysterols in murine models due to limited volume of materials available. Therefore, the GC/MS method using deuterium-labelled internal standards was developed, yielding high sensitivity analysis of 7-oxysterols and cholesterol and detecting concentrations in the mouse of a similar magnitude to those in previous literature reports from humans (Table 5 - 1). The method developed was suitable for measuring oxysterols in murine tissues and plasma and follows four key sample preparation steps:

- a) Solvent extraction*
- b) Solid-phase extraction (SPE)*
- c) Hydrolysis, and*
- d) 2<sup>nd</sup> Solvent extraction.*

*a) Solvent extraction*

Chloroform:methanol (2:1, v/v) (Sevanian *et al.*, 1994; Siems *et al.*, 2005) and diethylether:hexane (2:3, v/v) (Endo *et al.*, 2008) solvent extraction systems were utilized and recoveries compared as given in Table 5 - 2. Initial studies were carried out to investigate recovery of oxysterols from water and human plasma (300 – 1000 µl), allowing large volumes to be analysed in duplicate. Overall greater efficiency and minimum inter-assay variation of extraction was achieved using the mixture of diethylether in hexane.

*b) Solid phase extraction (SPE)*

The next stage involved solid phase extraction (SPE), which often uses silica (Si)- or diol (2-OH)-based columns in combination with different solvents (Simpson, 1992). Diol columns are suitable for processing of lipophilic biological materials and were suggested by Varian technical support. Diol columns were prepared with toluene:ethyl acetate (1:1, 2 ml), under vacuum as described (Endo *et al.*, 2008; Sevanian *et al.*, 1994) and used successfully before further detection of 7-oxysterols. Use of Si columns was abandoned, 7-oxysterols could not be detected in the eluate.

*c) Hydrolysis (Saponification)*

Saponification is an important step for isolation of sterols and oxysterols, which in biological tissues are usually present at much lower levels than triglycerides. This step is, therefore, employed prior to GC/MS to remove triglycerides, free fatty acids and water-soluble impurities during extraction of oxysterols. During this process, abundant lipids are hydrolyzed to form water-soluble components using NaOH or KOH (in methanol or ethanol). To avoid degradation of cholesterol and sterol oxides, a modification of previously reported methods (Brown *et al.*, 1997; Hodis *et al.*, 1991; Maerker and Unruh, 1986) was applied, involving very mild, ice-cold saponification (20 % KOH in methanol). Our findings agreed with previous studies, showing that higher saponification temperatures produce greater degradation of 7-KC (Park *et al.*, 1996; Schroepfer, 2000). For example Brown *et al.*, (1997) and Park *et al.*, (1996) achieved higher recovery of 7-KC (97%) with practically negligible loss after 18h of acid hydrolysis at RT, than after 30 min of heating (37°C, 45%; 75°C, 31%). Previously, data were not available concerning the recovery of 7 $\beta$ OHC, so tests were performed to check the effect of different temperature on hydrolysis. The highest abundance of both 7 $\beta$ OHC and 7-KC was achieved by performing the hydrolysis at 4 °C whereas at RT, 7 $\beta$ OHC had degraded more than 7-KC. These studies established that this step should be performed at 4°C.

*d) 2<sup>nd</sup> Solvent extraction.*

The second solvent extraction allowed recovery of 7-oxysterols after hydrolysis. Use of chloroform or diethylether resulted in reasonable and similar recoveries, however, diethyl ether solvent allowed quicker phase separation.

Based on the data given in the method development section, the reproducibility between the experiments was within acceptable limits of precision and accuracy, as particular care was taken to minimize the sample exposure to the air and pro-oxidative environment. To minimize this variation and prevent auto-oxidation, EDTA and argon were always used, as previously reported (Schroepfer, 2000). Since EDTA is able to quench metal ions that might contribute to auto-oxidation, EDTA coated onto vials and the collecting funnel, was used as the anti-coagulant for blood collection.

Levels of 7-oxysterols were consistently more readily detected in plasma and hepatic cytosol, than in aortic tissue, whilst an even greater abundance of 7-oxysterols was found in the hepatic microsomal preparations. For the chow-fed C57Bl/6 mice, it became apparent that plasma (~ 300 µl) and aortic samples from two animals had to be pooled for the robust detection of oxysterols. This was not the case for plasma used from ApoE<sup>-/-</sup> mice, in which cholesterol and oxysterols could be measured in a plasma volume between 150-250 µl. The method developed here can be applied successfully for detection of 7-oxysterols in 300 µl of plasma from normolipidaemic mice. New methods are constantly emerging for the detection of oxysterols in biological samples by liquid chromatography mass spectrometry (LC/MS) and novel derivatives are being developed to improve the sensitivity of detection. Comparisons of LC/MS with GC/MS have been assessed to compare sensitivity of these two approaches (Griffiths *et al.*, 2008; Honda *et al.*, 2008). Although the newly-developed method presented in this chapter provides sensitivity greater than HPLC-UV detection, the latest method for LC/MS detection by Honda *et al.*, (2008), to form picolinyl esters, may provide even greater sensitivity. These studies suggest using 100-300 µl of mouse plasma from normolipidemic mice for LC/MS detection

of 7-KC (DuSell et al., 2008; Umetani et al., 2007) as opposed to the 500  $\mu$ l volume previously recommended by others.

The analytical method developed was tested in pilot work using plasma from the normolipidemic 5 $\alpha$ SRD1<sup>+/-</sup> mice. These mice were used simply because they were being culled and, therefore, provided a ready supply of plasma. The concentrations of 7-oxysterols were established and in order of abundance were: 7-KC > 7 $\beta$ OHC > 7 $\alpha$ OHC. This pattern is consistent with previous studies measuring 7-oxysterols in human plasma (Iuliano *et al.*, 2003; Zieden *et al.*, 1999). In murine plasma, the levels of 7-oxysterols can vary between animals with different genetic backgrounds (Schroepfer, 2000; Terasaka *et al.*, 2007). Here, similar levels were detected in plasma from C57Bl/6 and 5 $\alpha$ SRD1<sup>+/-</sup> mice of a mixed (C57BL/6J//129Sv/Ev) background, while 5 to 9 times higher levels of 7-oxysterols were detected in plasma from ApoE<sup>-/-</sup> mice. Differences existed between the circulating levels of cholesterol and 7-KC between male and female mice. 7-KC levels were higher in female mice, whereas total cholesterol levels were higher in male mice. Gender differences have been reported for cholesterol (higher in females) or HDL cholesterol levels (higher in males), in murine models such as LDLR<sup>+/-</sup> or ApoE<sup>-/-</sup> (Bruell *et al.*, 1962; Lie *et al.*, 2006). Similar data have not always been clear for 7-oxysterols in human plasma (Dzeletovic et al., 1995b; Schroepfer, 2000), although the differences may simply be expected due to a different hormonal regulation of lipid metabolism between genders (Vitale *et al.*, 2009).

These preliminary experiments provided information on the range of concentrations of 7-oxysterols and un-esterified cholesterol in murine plasma. These concentrations were used to design further experiments testing the biological functions of 7-oxysterols on the vasculature. The ratio of 7-KC to 7 $\beta$ OHC was calculated in all further assays as a measure of net inter-conversion of the two 7-oxysterols. Gender differences were not considered in further work as only male mice were used for subsequent analysis.

### 5.5.2. Determination Of Lipid Levels In Animal Models

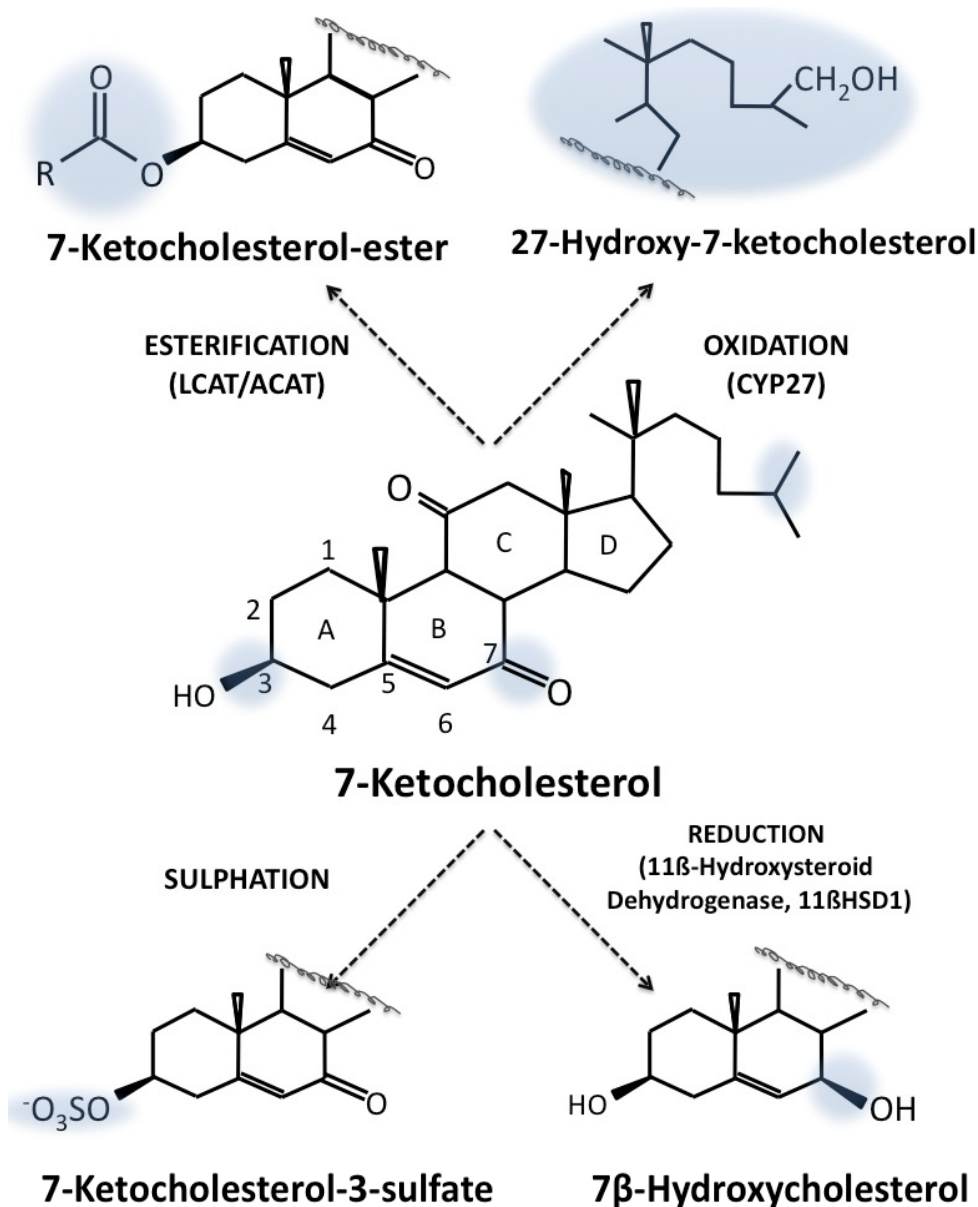
There are various factors that determine the levels of 7-oxysterol in the body, all of which may be subject to sexual dimorphism. Oxysterols are predominantly derived from the diet directly and/or by auto-oxidation of, or enzymatic synthesis (such as synthesis via 7 $\alpha$ -hydroxylase or 11 $\beta$ HSD1 (Brown and Jessup, 2009)), from cholesterol. To establish whether cholesterol is the major determinant of the levels of 7-oxysterols in biological fluids and plasma of mice, a correlation between 7-oxysterol levels with cholesterol levels was sought. There were positive correlations between 7-oxysterol and cholesterol levels in the plasma of C57Bl6 mice. However, in plasma of 11 $\beta$ HSD1<sup>-/-</sup> mice similar correlations were not observed. The explanation of this strain difference is not entirely clear although some explanations can be suggested. Previously, strong correlations were reported between total cholesterol concentrations and oxidized lipids (mainly 7-oxysterols) (Schroepfer, 2000). Katsumi *et al.*, (2000) reported a positive association between 7-KC and serum cholesterol concentrations in patients with poorly controlled type II diabetes *vs.* control patients. More recent work in adolescent girls indicates that serum oxysterol levels increase with LDL cholesterol, ApoB, insulin and obesity; all indicators of early stage metabolic syndrome (Alkazemi *et al.*, 2008). In contrast, there is also contradictory evidence in the literature (Schroepfer, 2000), including a study in healthy Italian volunteers, which demonstrated that neither 7-KC nor 7 $\beta$ OHC concentrations correlated with the total cholesterol concentrations, and suggested that production of oxysterols is not necessarily related to increased total cholesterol (Iuliano *et al.*, 2003). More recent study by the same research group speculated that the concentrations of circulating 7-oxysterol are influenced by enzymatic reactions, such as those investigated here, involving 11 $\beta$ HSD1 (Larsson *et al.*, 2007). The work in this chapter reports that upon disruption of 11 $\beta$ HSD1 in mice, 7-oxysterols do not correlate with cholesterol in plasma. There is not yet a plausible explanation as to why is this the case, although, possibly the reduction in cholesterol levels, but not oxysterols in these mice, might be the explanation. In addition, disruption of 11 $\beta$ HSD1 leads to an increase in circulating 7-KC despite the reduction in cholesterol.



It is however, possible that other metabolic routes of oxysterols may have changed in 11 $\beta$ HSD1<sup>-/-</sup> mice. This is likely given the changes in lipid-signalling pathways in the liver following over-expression of 11 $\beta$ HSD (Paterson *et al.*, 2004). This may disrupt the balance between the 7-oxysterol and cholesterol pools. The most obvious alternative route of 7-oxysterol metabolism in 11 $\beta$ HSD1<sup>-/-</sup> mice is via CYP27 (Figure 5.5 – 1), which could utilize 7-KC from plasma and liver (Li *et al.*, 2007). Extensive work by Freeman *et al.*, (2005) showed that formation of 7-ketocholesteryl esters as well as 7-KC-arachidonate accumulates in the macrophages and may represent a significant pool in these cells, leading to activation of cleaved caspase-3 (Freeman, 2005). Another widespread mechanism for metabolism of 7-oxysterols is sulphation of the 3 $\beta$ -hydroxyl group, thus in the absence of 11 $\beta$ HSD1, 7-KC is metabolized to 7KC-3-sulphate (Song *et al.*, 2001). Neither of these alternative metabolites were quantified in the assays used. Therefore, measurement of additional metabolites of oxysterols may be necessary to fully understand the relationship between cholesterol and oxysterols.

#### **5.5.2.1. 11 $\beta$ HSD1 Regulates The Levels Of 7-Oxysterols In Murine Plasma**

Although the levels of 7-oxysterols have been related to those of cholesterol in mice (Terasaka *et al.*, 2008), the significance of changes in ratios of individual oxysterols (7-KC and 7 $\beta$ OHC) in plasma has not been addressed. The proportions of 7-KC to 7 $\beta$ OHC were not considered in relation to the activity of 11 $\beta$ HSD1 before. Steffen *et al.*, (2006) have investigated the proportions of 7 $\beta$ OHC to 7-KC in oxidized LDL (oxLDL). Their work showed that the ratio of 7 $\beta$ OHC to 7-KC in a mixture of oxysterols, is a crucial determinant of the cytotoxicity of oxLDL to endothelial cells. The importance of the balance between individual 7-oxysterols has also been alluded to in a study by Breuer *et al.*, (1993) who measured 7-KC and 7 $\beta$ OHC in rat liver, proposing that reduced levels of 7-KC may be associated with a rise in the levels of 7 $\beta$ OHC. This finding suggested that formation of 7 $\beta$ OHC occurs from 7-KC, although they were not aware of the 11 $\beta$ HSD1-mediated metabolism of these species at the time.



**Figure 5.5-1 Routes of metabolism of 7-ketocholesterol (7-KC)**

Possible routes of metabolism of 7-KC in rodents and primates. Reaction with 7-KC can take place on the A or B rings or on the side-chain of the cholesterol backbone. CYP27, Cytochrome P450 27-hydroxylase; LCAT, Lecithin : cholesterol acyl-transferase; ACAT, Acyl-coenzyme A : cholesterol acyltransferase.

Therefore from the perspective of Breuer *et al.*, inhibition of 11 $\beta$ HSD1 could contribute towards accumulation of 7-KC and reduction in 7 $\beta$ OHC levels in the plasma and peripheral tissues. In this chapter, two models were used to examine the effect of inhibition of 11 $\beta$ HSD1 on the abundance of 7-oxysterols: 11 $\beta$ HSD1 knockout mice and administration of a pharmacological inhibitor of 11 $\beta$ HSD1. In the plasma of 11 $\beta$ HSD1<sup>-/-</sup> mice there was a trend towards higher concentrations of 7-KC, and the ratio of 7-KC/7 $\beta$ OHC was higher compared to C57Bl/6 mice. The pronounced increase in 7-KC would be expected upon disruption of 11 $\beta$ HSD1 if reduction of 7-KC to 7 $\beta$ OHC was the predominant route of metabolism in vivo. The data from this work suggest this may still be the case, although the in vitro findings in Chapter 3 of this thesis showed that 11 $\beta$ HSD1 was a predominant dehydrogenase for 7-oxysterols. The disruption of 11 $\beta$ HSD1 led to reduced levels of 7-oxysterols in hepatic microsomes and cytosols, thus demonstrating that 11 $\beta$ HSD1 determines the cellular levels of these compounds. If this is the case in vivo, an increase in plasma levels of 7 $\beta$ OHC should be anticipated following disruption of 11 $\beta$ HSD1.

When levels of 7-oxysterols were normalized to the total cholesterol the effect of 11 $\beta$ HSD1 disruption became evident, in that both 7-KC/Chol and 7 $\beta$ OHC/Chol were increased. Since this finding was established for both 7-KC and 7 $\beta$ OHC, it is not possible to hypothesize that 11 $\beta$ HSD1 only catalyses initial step of the detoxification of 7-KC, reducing it stereospecifically to 7 $\beta$ OHC. Perhaps, the oxidation of 7 $\beta$ OHC to 7-KC by 11 $\beta$ HSD1 occurs simultaneously in the body, or there are other sources of 7 $\beta$ OHC in the 11 $\beta$ HSD1<sup>-/-</sup> mice, like reactive oxygen species (ROS) production and lipid oxidation (Rimner *et al.*, 2005).

Levels of 7-oxysterols in hyperlipidaemic mice are within the same order of magnitude ( $\mu$ M) as the  $K_m$  of 7 $\beta$ OHC. Therefore, changes in the balance of 7-oxysterols are perhaps of low significance for metabolism of glucocorticoids by 11 $\beta$ HSD1 in the animals fed chow diet, but may be of greater importance in mice fed a high fat diet, and power the metabolism of 7-oxysterols by 11 $\beta$ HSD1. It is assumed that increased circulatory levels of 7-oxysterols may also be delivered into tissues where, through nuclear signalling or direct molecular interaction, they may impair

tissue function (Deckert et al., 2002; Deckert et al., 1997). Translated into hyperlipidaemic (e.g. ApoE<sup>-/-</sup> or LDLR<sup>-/-</sup>) mice, where increased levels of 7-oxysterols are observed (Brown and Jessup, 1999), 11 $\beta$ HSD1 may be balancing the ratio of 7-KC to 7 $\beta$ OHC and thus influence the magnitude of their proatherogenic actions.

#### **5.5.2.2. 11 $\beta$ HSD1 Regulates The Levels of 7-Oxysterols In Murine Hepatic Fractions**

In contrast to plasma ratios of 7-KC/7 $\beta$ OHC, this ratio was reduced (compared with C57Bl/6 controls) in hepatic microsomes from 11 $\beta$ HSD1<sup>-/-</sup> mice. This is consistent with a predominant dehydrogenation of 7-oxysterols by 11 $\beta$ HSD1. The reason for different profiles of 7-oxysterols between plasma and liver is not clear, as mechanisms for packaging 7-oxysterols into plasma lipoproteins or delivery of 7-oxysterols to the ER lumen are not well understood. However, the microsomal compartment is intimately linked to 11 $\beta$ HSD1 expression and it is perhaps not surprising that more profound differences are observed here. Similar amounts of 7-oxysterols were seen in microsomes and cytosols from C57Bl/6 mice, but became almost undetectable in the cytosols of 11 $\beta$ HSD1<sup>-/-</sup> mice, suggesting the 7-oxysterols are anchored in some way within this compartment. Overall, reduced levels of 7-oxysterols in the microsomes of 11 $\beta$ HSD1<sup>-/-</sup> mice are accompanied by lowered total cholesterol levels. The microsomal ratio of 7-KC/7 $\beta$ OHC was significantly reduced due to disruption of 11 $\beta$ HSD1, whereas this ratio could not be established for cytosols from 11 $\beta$ HSD1<sup>-/-</sup> mice. It is, thus, likely that expression of 11 $\beta$ HSD1 influences the cellular distribution of hepatic 7-oxysterols.

### **5.5.2.3. Pharmacological Inhibition of 11 $\beta$ HSD1 Does Not Inhibit Metabolism of 7-Oxysterols**

The role of 11 $\beta$ HSD1 in metabolism of 7-oxysterols was also explored pharmacologically. The activity of the 11 $\beta$ HSD1 inhibitor (compound 544) was confirmed by the demonstration that it inhibited the conversion of glucocorticoids in cultured cells and reduced lesion size in atherosclerotic mice (Iqbal *et al.*, 2009), similar to reports by Hermanowski-Vosatka *et al.*, (2005). However, compound 544 did not inhibit the metabolism of 7-oxysterols by 11 $\beta$ HSD1 in cultured cells. Hepatic levels or proportions of 7-oxysterols were not altered in the hyperlipidaemic ApoE<sup>-/-</sup> mice and ApoE<sup>-/-</sup> mice administered the 11 $\beta$ HSD1 inhibitor (compound 544) (Iqbal *et al.*, 2009), suggesting either low efficacy of drug delivery to liver compartments, or selective inhibition of glucocorticoid metabolism. The levels of 7-oxysterols and cholesterol show variation within the same group but the difference between treatments was not seen, even after normalizing the levels of 7-oxysterol to the total cholesterol. A possible explanation may lie in time of sampling, since expression of various genes for the enzymes in hepatic metabolic pathways are under the circadian expression and a control of multiple mechanisms including the CLOCK molecule (Noshiro *et al.*, 2007). Thus the levels of circulating 7-oxysterols might have varied within treated and control groups depending on the time of blood collection (am or pm) due to diurnal variation in peripheral tissues (Reilly *et al.*, 2007).

More likely elucidation for the lack of inhibitory effects on metabolism of 7-oxysterols, may lie in the design of compound 544. Since metabolism of glucocorticoids by 11 $\beta$ HSD1 was still dimly observed in the presence of the inhibitor, it would appear that compound 544 does not mask enzyme's active site or completely abolish binding of substrates. This latter finding concurs with the data established in this chapter, using the inhibitor with HEK293 cells *in vitro*, in which glucocorticoid (but not 7-oxysterol) metabolism by 11 $\beta$ HSD1 was altered. Indeed, this inhibitor acts to specifically prevent glucocorticoids from accessing the active site, while inadvertently favouring the binding of 7-oxysterols. Therefore, clear differences have been established in this chapter, between the interactions of

11 $\beta$ HSD1 inhibitor (compound 544) with glucocorticoid versus 7-oxysterol substrates in the active site of 11 $\beta$ HSD1 to inhibit the activity of this enzyme.

#### 5.5.2.4. **Levels Of 7-Oxysterols In Aortae Of C57Bl/6 Mice**

Assessment of 7-oxysterols in the peripheral tissue, namely the aorta, was performed using C57BL/6 mice. In the aorta of C57Bl/6 mice fed chow diet, levels of oxysterols were relatively low but were, nevertheless, detectable and 7-KC levels were greater than 7 $\beta$ OHC. In a recent study, Umetani *et al.*, (2007) detected similar levels of 7-KC and 27-OHC in murine aorta to those reported here, but found that the levels increased three times when mice were placed on a high cholesterol high fat (HCF) diet. Previous reports in humans show that levels of 7-KC and 7 $\beta$ OHC in atherosclerotic plaque increase with progression of atherosclerosis (Brown *et al.*, 1997; Vaya *et al.*, 2001) and this has also been reported for ApoE<sup>-/-</sup> mice (Brown and Jessup, 1999). It may be expected for both 7-KC and 7 $\beta$ OHC to accumulate in the vessels, in atherosclerosis (Thomas *et al.*, 2001). Only one other study reported on oxysterol levels in the vessel wall. Findings by Terasaka *et al.*, (2008) reported that 7-KC levels were undetectable in the aortae of chow fed mice, but could be measured in the aortae of mice fed high cholesterol diet. This is certainly in line with results presented in this chapter.

Consistent with results obtained using plasma, levels of 7-KC were higher than 7 $\beta$ OHC in the aorta, perhaps suggesting that the main source of vascular oxysterols is from the plasma pool. However, levels of 7-KC were ten fold higher than those of 7 $\beta$ OHC in vessels as opposed to two fold higher in plasma. As such, the ratio of 7-KC/7 $\beta$ OHC was higher in the vessels than in microsomes, cytosols or plasma. The mechanisms of oxysterol inactivation and clearance in the vessel are not known. Accumulation of 7-KC via 11 $\beta$ HSD1-mediated dehydrogenation of 7 $\beta$ OHC may subsequently result in the 7-KC being sequestered more readily by the endothelium, smooth muscle cells and other surrounding cells (macrophages), as reported before (Kritharides, 1995; Santillan *et al.*, 1982; Tall *et al.*, 2008). The macrophages within the vessel wall may also contain a set of enzymes for further processing of oxysterols

prior to or after their storage. It is possible that 7 $\beta$ OHC is metabolised or exported at a much faster rate than 7-KC by the vasculature. Differences in storage of 7-KC versus metabolism of 7 $\beta$ OHC, may therefore contribute to an increase in the ratio of 7-KC/7 $\beta$ OHC in the vessels. Therefore, the balance of export and metabolism of 7-oxysterols is clearly different between the aortae and the liver.

The metabolism of 7-oxysterols has so far been best described in the liver, where clearance of 7-KC occurs relatively quickly (Erickson *et al.*, 1977; Lyons *et al.*, 1999), by a rapid transfer from chylomicrons to other lipoprotein and/or plasma fraction (Emanuel *et al.*, 1991). 7-KC can be converted into bile acids for excretion out of the body (for review see Brown *et al.*, (1999) and Carpenter (2002)) or be metabolised by various aldo-keto reductases specific to the liver (e.g. 3 $\alpha$ -hydroxysteroid dehydrogenase (3 $\alpha$ -HSD) or CYP450s, which are not present on the peripheries (Jin and Pennings, 2007; Qin and Cheng, 1994)). The evidence for other route of metabolism of 7-oxysterols outside of liver comes from a study in patients with cerebrotendinous xanthomatosis (CTX), who lack 27-hydroxylase (CYP27), (Cali *et al.*, 1991; Fujiyama *et al.*, 1991). Patients with CTX accumulate 7-KC in the macrophages and in plasma due to their inability to further metabolise 7-KC. In the macrophages from normal patients, CYP27 reduces 7-KC to 27-hydroxy-7-ketocholesterol (27OH-7-KC) (Figure 5.5 – 1). However, it is suggested by Jessup *et al.*, (2005) that high levels of 7-KC in patients with CTX are actually lower than expected, because in the macrophages 11 $\beta$ HSD1 also contributes to the clearance of 7-KC. It is not clear from the literature whether 7 $\beta$ OHC can also undergo this type of metabolism. It is however tempting to speculate that the formation of 27-hydroxy-7 $\beta$ OHC can occur via CYP27 route too. This would suggest that efficient metabolism of 7-KC includes reduction to 7 $\beta$ OHC by 11 $\beta$ HSD1, then formation of 27-hydroxy-7 $\beta$ OHC by CYP27 and further metabolism to bile acids. Being that 7 $\beta$ OHC is relatively short-lived (1.5h) (Larsson *et al.*, 2007), it may more readily be inactivated by auto-oxidation (Santillan *et al.*, 1980). Thus, high levels of 7-KC and ratio of 7-KC/7 $\beta$ OHC observed in the normolipidaemic aortae in this chapter may indeed be cleared out via 11 $\beta$ HSD1 and CYP27 enzymes. However, the activity of 11 $\beta$ HSD1 (not tested for CYP27 here) is much slower in the vessels compared to the

liver, and thus clearance of 7-KC in this tissue occurred at a slower rate, measuring high 7-KC.

In summary, the work in this chapter provides evidence that 11 $\beta$ HSD1 controls aortic, hepatic and plasma level of 7-oxysterol in normolipidemic mice. Levels of 7-oxysterols are elevated in plasma and tissues with development of atherosclerosis, but current available 11 $\beta$ HSD1 inhibitor is not designed to reduce the metabolism of 7-oxysterols, or their levels and proportions in murine tissues. Increased 7-oxysterols, may thus compete with glucocorticoids and inhibit dehydrogenation by murine 11 $\beta$ HSD1, to foster the formation of active glucocorticoid in atherosclerosis.



## **Chapter 6. Impact Of 7-Oxysterols On Function Of Murine Aortae**

## 6.1. Introduction

Previous studies of 11 $\beta$ HSD1-mediated metabolism of glucocorticoids in vascular tissue have demonstrated the presence of both isozymes of 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ HSD1 and 11 $\beta$ HSD2) in vascular cells (Christy *et al.*, 2003; Krozowski and Chai, 2003; Walker *et al.*, 1991). Controversy still exists concerning the localisation and directionality of these enzymes in the vascular wall (Morris *et al.*, 2003). It is generally accepted, however, that (in murine aorta, at least) 11 $\beta$ HSD1 is localized predominantly to infiltrated macrophages and vascular smooth muscle cells (VSMC) (Cai *et al.*, 2001; Hatakeyama *et al.*, 2001). In contrast, 11 $\beta$ HSD2 is restricted to the endothelial cells (Christy *et al.*, 2003). In the vascular wall both isozymes of 11 $\beta$ HSD actively interconvert glucocorticoids (Dover *et al.*, 2006).

The work in Chapter 5 presented evidence that 7-ketocholesterol (7-KC) may be present in relatively high levels compared with 7 $\beta$ -hydroxycholesterol (7 $\beta$ OHC) in the vessel wall. This may have important implications for vascular function as an increase in the oxidized lipid content of endothelial cells has previously been shown to inhibit the endothelium-dependent relaxation of aortic segments from hyperlipidaemic mice (Deckert *et al.*, 1999). 7 $\beta$ -Hydroxycholesterol and 7-ketocholesterol can mimic the ability of oxidized lipids (oxLDL) to inhibit the relaxation response to acetylcholine, as shown in rabbit aortic segments *in vitro* (Deckert *et al.*, 1997). The molecular mechanism of these inhibitory effects on arterial relaxation is, as yet, unknown and indeed differences between the vascular effects of these two oxysterols poorly characterised. Alterations in endothelium-dependent relaxation by 7-oxysterols have been found to be independent of their known cytotoxic effects (Lizard *et al.*, 1997), but in HUVECs 7 $\beta$ OHC and 7-KC reduced the histamine-activated release of nitric oxide (Deckert *et al.*, 1998).

The work described in Chapter 3 would predict that 11 $\beta$ HSD1 in the vessel wall can interconvert 7-oxysterols. Thus, it has been proposed that 11 $\beta$ HSD1 may regulate the concentrations of 7-KC and 7 $\beta$ OHC in the arterial wall. Whether 11 $\beta$ HSD1-mediated metabolism of 7-oxysterols in vascular cells influences the functional consequences

of exposure of vessels to these compounds, has not yet been determined. This chapter investigates the direct influence of 7-KC and 7 $\beta$ OHC on the function of murine aortae and assesses the ability of vascular 11 $\beta$ HSD1 to interconvert these 7-oxysterols.

## 6.2. Research Hypothesis And Aims

This chapter explores the hypothesis that 7-oxysterols generated by 11 $\beta$ HSD1 in the vascular wall impair functional responses of the murine aortae *ex vivo*.

- The specific aims for this chapter were to:
- Develop a method to allow investigation of the effects of 7-oxysterols on vascular function, and;
- Determine whether incubation with 7-KC or 7 $\beta$ OHC *ex vivo* alters the function of isolated murine thoracic aortae.

## 6.3. Methods

### 6.3.1. Method Development

The experiments described in this chapter were designed to test the functional effects of exposure to 7-oxysterols on murine thoracic aortae *ex vivo*. This required extended incubation of isolated vessels, raising concerns over preservation of vascular function and prompting pilot experiments to determine whether use of tissue culture medium (DMEM) was preferable to physiological saline solution (PSS). Previous work by Christy *et al.*, (2003) of *ex vivo* incubations of aorta suggested that DMEM preserves vascular function after extended periods of incubation, but certain constituents of DMEM (such as L-arginine) have unwanted vasoactive properties (Christy *et al.*, 2003). Therefore, for the purpose of work presented in this thesis DMEM without L-Arg was used, and outcomes were described further in method development, section 6.4.1. To determine whether functional experiments could be performed in arteries bathed in culture medium, contractile and relaxant responses

were compared in aortic rings (male C57Bl/6 mice, age 8-12 weeks, n=4) in DMEM without L-arginine (DMEM-L-Arg) or in PSS.

#### **6.3.1.1. Preparation of Solution of 7-Oxysterols**

Stock solutions of 7-oxysterols (30 mg/ml in ethanol) were freshly prepared as described (section 2.2.2), including butylated hydroxytoluene (BHT; 250 µg/ml) as an antioxidant (Schroepfer, 2000). Working solutions were prepared by adding 7-oxysterol stock (60 µl) to culture medium (DMEM-L-Arg; 30 ml, containing 1% v/v stripped FBS, as in section 2.6.1.1) to achieve working concentrations of 7-KC (25 µM) or 7βOHC (20 µM) (Lizard *et al.*, 1999; Seye *et al.*, 2004). Samples were vortexed (400 rpm, RT, 10 min) then sonicated (10 min, RT). The final concentration of vehicle (ethanol with 250 µg/ml BHT) in DMEM was < 0.2%.

#### **6.3.1.2. Does Culture Medium Alter The Functional Responses Of Aortic Rings From C57Bl/6 Mice?**

Thoracic aortae from C57Bl/6 mice (male, age 8 - 12 weeks, n=12) were collected in PSS or culture medium (DMEM) and dissected into four rings (section 2.4.5), which were mounted in a small vessel wire myograph (section 2.10.1). Endothelium was removed from the aortic rings by rubbing with a wire probe (section 2.10.2). The rings were incubated under optimal resting tension (7.36 mN) (Bagnall *et al.*, 2006) in either PSS or DMEM-L-Arg at 37°C with 95% O<sub>2</sub>/ 5% CO<sub>2</sub>. The aortic rings were then subjected to the standard experimental protocol and vasorelaxation and vasocontraction were measured as described (section 2.10).

#### **6.3.1.3. Functional Responses Of Aortae From 11βHSD1<sup>-/-</sup> Mice: Effect Of Endothelium**

To determine whether aortic rings from 11βHSD1<sup>-/-</sup> mice on a C57Bl/6 background produced the expected responses when assayed in culture medium, the effect of endothelial removal from these vessels was assessed using DMEM-L-Arg rather than PSS. Thoracic aortae from 11βHSD1<sup>-/-</sup> mice (male, age 8-12 weeks, n=6) were collected and dissected in sterile DMEM-L-Arg, and divided into four rings of which

two had the endothelium removed. The standard experimental protocol (section 2.10.3) was performed to confirm the pattern of functional responses of aortic rings with and without the endothelium.

### **6.3.2. Effect Of 7-Oxysterols On Vascular Function *In Vitro***

To determine whether exposure to 7-oxysterols altered aortic contraction or relaxation, arteries from C57Bl/6 mice were subjected to short (4h) or long (24h) exposure to 7-oxysterols (7-KC or 7 $\beta$ OHC) or vehicle in DMEM-L-Arg prior to functional analysis. The possibility that 11 $\beta$ HSD1 in the isolated aorta could metabolise 7-oxysterols in the incubation mixture was assessed by measuring concentrations of both 7-KC and 7 $\beta$ OHC at the end of the incubation period.

#### **6.3.2.1. Short-Term Exposure Of Aortic Rings 7-Keto-cholesterol And 7 $\beta$ -Hydroxycholesterol**

Thoracic aortae from C57Bl/6 mice (male, age 8 - 12 weeks, n=8) were divided into four rings and submitted to the standard experimental protocol (section 2.10.3). The vessels were equilibrated in oxygenated DMEM-L-Arg (95% O<sub>2</sub> and 5% CO<sub>2</sub>, 37°C) containing stripped FBS (1% v/v). Cumulative concentration-response curves were then obtained using the vasoconstrictors noradrenaline (10<sup>-9</sup> – 10<sup>-5</sup> M), serotonin (5HT; 10<sup>-9</sup> – 10<sup>-4</sup> M) and potassium chloride (10 – 125 mM). Aortic rings were further equilibrated (30 min), then incubated with 7-KC (25  $\mu$ M, one ring) or 7 $\beta$ OHC (20  $\mu$ M, one ring) in DMEM-L-Arg and two rings in vehicle only (EtOH, 50  $\mu$ g/ml BHT) for 4 hours (incubation medium was refreshed every 2h) and further functional responses were measured, as in section 6.3.2.4.

#### **6.3.2.2. Long-Term Exposure Of Aortic Rings To 7-Keto-cholesterol And 7 $\beta$ -Hydroxycholesterol**

To further assess the effects of 7-oxysterols on vascular function, aortic rings were prepared from aortae of C57Bl/6 mice (male, age 8-12 weeks, n=6) as in section 6.3.2.1, for an overnight incubation (24 h) in a humidified atmosphere (95% O<sub>2</sub> / 5% CO<sub>2</sub>, 37°C, 24 h). Single aortic rings were immersed in DMEM-L-Arg containing 7-

KC (25  $\mu$ M), 7 $\beta$ OHC (20  $\mu$ M), or vehicle (EtOH, 50  $\mu$ g/ml BHT) in a 12-well plate. Following incubation, vessels were mounted onto the myograph and subsequent functional responses were measured (section 6.3.3.4).

#### **6.3.2.3. Does Vascular 11 $\beta$ HSD1 Metabolise 7-Oxysterols in Isolated Aortic Rings?**

To determine whether 11 $\beta$ HSD1 in the aortic ring metabolises 7-oxysterols during the incubation period, samples (2 ml) of incubating solutions were collected after the incubation procedure (4 h and 24 h). Oxysterols were extracted from the medium (section 2.6.3.1) and detected using GC/MS analysis (section 2.13) to assess both stability and the extent of conversion by 11 $\beta$ HSD1 (section 6.4.2.3).

#### **6.3.2.4. Measurement Of Vascular Responses**

At the end of the prescribed incubation period, and in the continued presence of the 7-oxysterol or control solution, cumulative concentration-response curves were constructed for 5HT ( $10^{-9}$  –  $10^{-4}$  M) and NA ( $10^{-9}$  –  $10^{-4}$  M). In addition, cumulative concentration-response curves were performed for the vasodilators, ACh ( $10^{-9}$  –  $10^{-2}$  M, endothelium-dependent) and SNP ( $10^{-9}$  –  $10^{-4}$  M; endothelium-independent), following contraction with a sub-maximal concentration ( $EC_{80}$ ,  $3 \times 10^{-7}$  –  $10^{-6}$  M) of 5HT.

#### **6.3.3. Data Analysis**

Mac Lab Chart version 3.3 was used to record all myography data recording at a speed of 20 or 10 divisions/s. All analyses were performed using Graph Pad Prism v5.0 software by fitting Hill curves. Contractile responses are expressed as force per unit length (mN/mm) and as a percentage of the maximum contraction in response to KPSS (% KPSS). Relaxations were expressed as a percentage of the contraction in response to the  $EC_{80}$  of 5HT (% 5HT).

**Sensitivity** is a negative logarithm of the molar concentration producing a half-maximal relaxation ( $IC_{50}$ ) or contraction effects ( $EC_{50}$ ) and is hence expressed as **-Log $IC_{50}$**  for vasorelaxants, and **pD<sub>2</sub> (-Log $EC_{50}$ )** for vasoconstrictors. Sensitivity values were obtained after applying the sigmoid dose-response equation:  **$Y = Bottom + (Top - Bottom) / (1 + 10^{-(LogEC_{50} - X)})$** ; where  $X$  is logarithm of concentration,  $Y$  is the response.  $E_{max}$  is the maximal response to a drug. All maximal relaxations ( $E_{max}$ ), induced either by ACh or by SNP, were expressed as a percentage to 5HT (0.3  $\mu$ M) from experimental data.

#### **6.3.4. Statistical Analysis**

For all experiments using animal tissues, individual experiments were carried out using aortae from 4 - 14 mice. Where two denuded and two intact rings were used from individual aortae, data were calculated by obtaining an average result from the two comparable (i.e. with endothelium or without endothelium) aortic rings from each animal. All data are mean  $\pm$  standard error of the mean (SEM) and were compared using unpaired Student's t-test or 1-way ANOVA with Tukey's multiple comparison post-test as appropriate. P values were accepted as statistically significant when  $<0.05$ ; n represents the number of different animals used.

## 6.4. Results

The experiments described in this chapter were designed to test the effects of 7-KC and 7 $\beta$ OHC on the function of mouse aorta. Prior to these experiments, preliminary work was performed to establish suitable conditions for incubation of vessels with 7-oxysterols. Endothelium-intact and -denuded aortic rings were compared to demonstrate the functional consequences of severe endothelial dysfunction. Myography experiments were subsequently performed to test whether 7-oxysterols inhibit vascular contraction in response to 5HT, NA and KPSS, or endothelium-dependent/-independent relaxation, in response to ACh/SNP. Levels of 7-KC and 7 $\beta$ OHC were measured in the medium following vessel incubation, to determine whether metabolism of 7-oxysterols by 11 $\beta$ HSD1 has occurred.

### 6.4.1. Method Development

#### 6.4.1.1. *Preparation of Solutions of 7-Oxysterols*

The highest concentrations of 7-oxysterols that could be maintained in solution in DMEM were 25  $\mu$ M (7-KC) and 20  $\mu$ M (7 $\beta$ OHC). Preparation of higher concentrations (Deckert *et al.*, 2002) led to precipitation of the oxysterols.

#### 6.4.1.2. *Incubation Of Vessels In PSS And DMEM*

To determine whether functional investigations could be performed using culture medium in the myograph bath, cumulative concentration-response curves were produced for 5HT, NA, ACh and SNP in vessels bathed in PSS or DMEM-L-Arg. No difference in maximal response was observed during the general experimental protocol (section 2.10.3) with KPSS but contractions appeared slower to develop in arteries bathed in DMEM-L-Arg. As expected, mechanical removal of the endothelium was associated with a smaller response to KPSS (Table 6 - 1A) and virtual abolition of acetylcholine-mediated relaxation.



Both 5HT and NA produced concentration-dependent contractions in intact and denuded aortic rings. Bathing the vessels in DMEM-L-Arg had no significant effect on the size ( $E_{\max}$ , Table 6 - 1A) or sensitivity ( $pD_2$ , Table 6 - 1B) of the responses produced. A trend towards reduced maximal response to NA for vessels bathed in DMEM-L-Arg did not achieve significance ( $p=0.05$ ). In arteries bathed in DMEM-L-Arg, calculation of contractile response as a percentage of the maximum response to KPSS indicated that removal of the endothelium produced an increased  $E_{\max}$  to NA but not to 5HT (Figure 6.4-1).

Maximum response ( $E_{\max}$ ) and sensitivity ( $-\log IC_{50}$ ) to ACh or SNP were not different if aortic rings were bathed in DMEM-L-Arg rather than PSS (Table 6 - 1 A & B). As expected, ACh-induced relaxation was attenuated but SNP-mediated relaxation unaffected by removal of the endothelium whether the aortic rings were bathed in PSS (Table 6 - 1) or in DMEM-L-Arg (Figure 6.4 – 1).

**Table 6-1A Maximal contraction and relaxation of isolated mouse aorta are not altered if myography is performed using culture medium rather than PSS.**

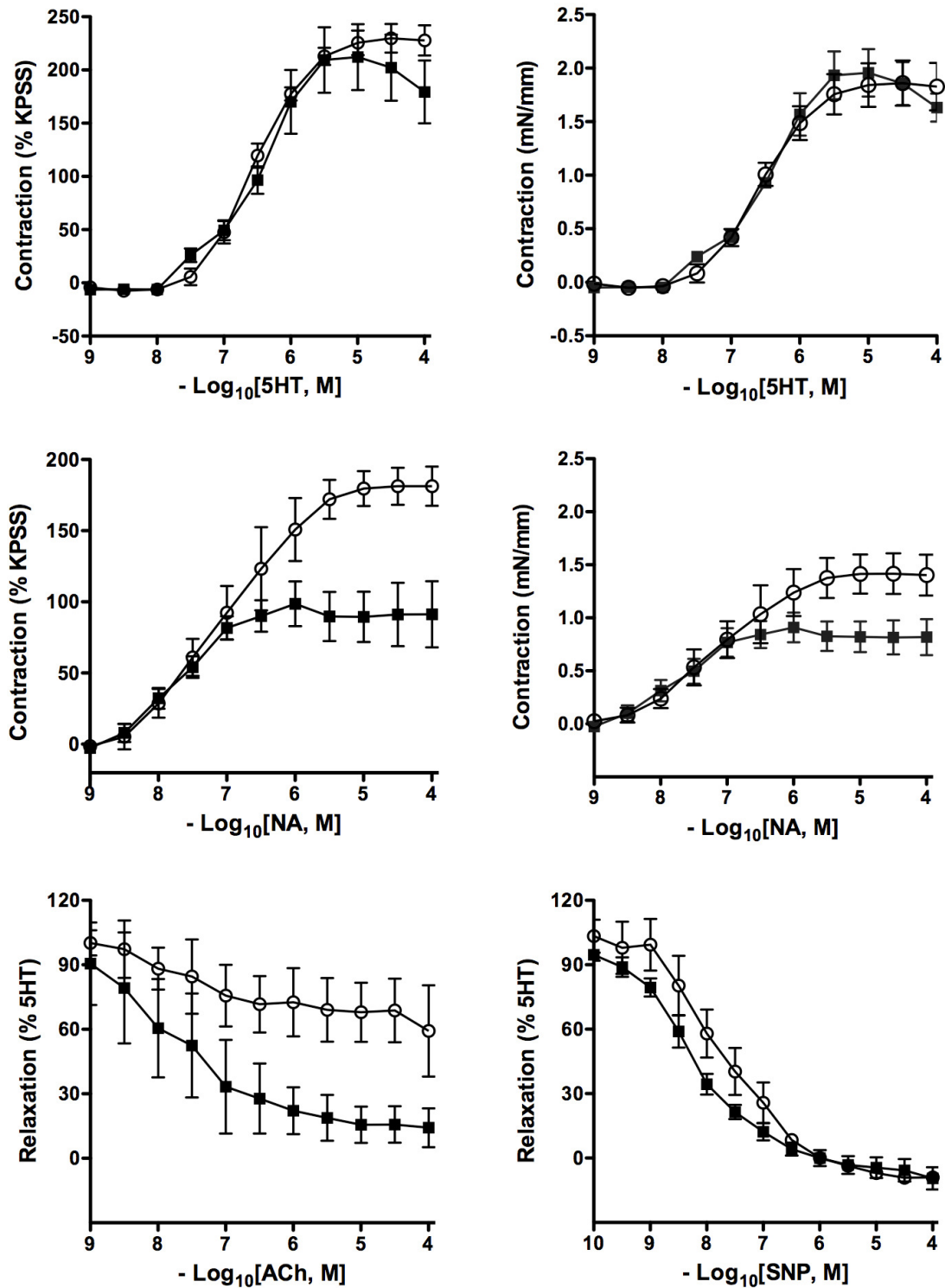
Table 6-1 A		$E_{\max}$		p-value
Drug	Endothelium	PSS	DMEM-L-Arg	
5HT	Intact	$2.63 \pm 0.22$	$2.40 \pm 0.43$	0.69
	Denuded	$2.25 \pm 0.69$	$2.22 \pm 0.22$	0.88
ACh	Intact	$79.90 \pm 5.66$	$64.20 \pm 6.34$	0.11
	Denuded	not calculated	not calculated	
NA	Intact	$1.21 \pm 0.18$	$0.96 \pm 0.15$	0.05
	Denuded	$1.99 \pm 0.74$	$1.44 \pm 0.19$	0.48
SNP	Intact	$100.53 \pm 0.41$	$102.80 \pm 2.68$	0.34
	Denuded	$108.71 \pm 3.70$	$113.54 \pm 3.04$	0.48

Maximal ( $E_{\max}$ ) relaxation (%) and contraction (mN/mm) responses evoked by vasoconstrictors (5HT, NA) and vasodilators (ACh, SNP) in the vessels bathed in either physiological salt solution (PSS) or culture medium (DMEM-L-Arg). Data were obtained using intact and denuded rings of thoracic aortae isolated from adult, male, C57Bl/6 mice. The use of DMEM-L-Arg did not significantly alter the maximum functional responses produced by mouse aortae. All values represent mean  $\pm$  SEM, compared by an unpaired Student's t-test,  $n=4$ ,  $p<0.05$ . NA, noradrenaline; 5HT, 5-hydroxytryptamine; ACh, acetylcholine; SNP, sodium nitroprusside; p-values are obtained upon comparison between vessels bathed in culture medium and PSS.

**Table 6-1B Sensitivity of isolated mouse aortic rings to vasoactive factors is not altered if myography is performed using culture medium rather than PSS.**

Table 6-1 B	Sensitivity			p-value
Drug	Endothelium	PSS	DMEM-L-Arg	
5HT	Intact	6.93 ± 0.12	6.60 ± 0.09	0.07
	Denuded	7.01 ± 0.22	6.54 ± 0.10	0.11
ACh	Intact	6.84 ± 0.37	8.17 ± 0.83	0.19
	Denuded	not calculated	not calculated	
NA	Intact	6.41 ± 1.19	7.85 ± 0.25	0.16
	Denuded	8.04 ± 0.24	6.98 ± 0.32	0.81
SNP	Intact	7.61 ± 0.17	8.23 ± 0.16	0.15
	Denuded	8.30 ± 0.17	7.71 ± 0.22	0.08

Sensitivity ( $pD_2$  or  $-\log IC_{50}$ ) of functional responses evoked by vasoconstrictors (5HT, NA) and vasodilators (ACh, SNP) in vessels bathed in either physiological salt solution (PSS) or culture medium (DMEM-L-Arg). Data were obtained using intact and denuded rings of thoracic aortae isolated from adult, male, C57Bl/6 mice. The use of DMEM-L-Arg did not significantly alter the sensitivity of responses to any of the compounds. All values represent mean ± SEM, compared by unpaired Student's t-test,  $n=4$ ,  $p<0.05$ . NA, noradrenaline; 5HT, 5-hydroxytryptamine; ACh, acetylcholine; SNP, sodium nitroprusside; p-values are obtained upon comparison between vessels bathed in culture medium and PSS.



**Figure 6.4-1 Cumulative concentration-response curves obtained from aortic rings bathed in DMEM without L-arginine.**

In the endothelium intact aortic rings (black squares) from male C57Bl/6 mice bathed in culture medium (DMEM-L-Arg) the removal of the endothelium (open circles) had no effect on 5-hydroxytryptamine (5HT)-mediated contraction, but it has enhanced contraction to

noradrenaline (NA). Presentation of the data as a percentage of the response to physiological salt solution with potassium (KPSS) corrects for damage to the medial smooth muscle in denuded arteries. Acetylcholine (ACh)-mediated relaxation, but not the response to sodium nitroprusside (SNP), was virtually abolished by removal of the endothelium. These results are consistent with the effects of denudation obtained in arteries bathed in PSS. All points represent mean  $\pm$  SEM, compared by 1-way ANOVA with Tukey's post hoc test, n=4.

#### **6.4.1.3. Effect Of Endothelium On Aortae From 11 $\beta$ HSD1<sup>-/-</sup> Mice**

Vascular reactivity was measured in intact and denuded aortic rings from 11 $\beta$ HSD1<sup>-/-</sup> mice in bathed DMEM-L-Arg. As anticipated, removal of the endothelium had a tendency to reduce the contractile response to 125 mM K<sup>+</sup> (KPSS; Table 6 - 2). The maximal response ( $E_{max}$ ), but not the sensitivity ( $pD_2$ ), to 5HT was reduced in endothelium-denuded, compared with endothelium-intact, aortic rings ( $p \leq 0.02$ ). However, when the  $E_{max}$  was expressed as a percentage of the response to KPSS (to correct for mechanical injury to the vessel wall), it was shown that denudation resulted in an enhanced response to 5HT (Table 6 - 2). A similar effect of endothelial cell removal was seen with the response to NA.

Removal of the endothelium virtually abolished the relaxant response to ACh but caused a significant ( $p=0.02$ ) increase in the relaxant response to SNP (Table 6 - 2). The sensitivity of the tissue to SNP, however, was unchanged.

**Table 6-2 The impact of removal of endothelial cell on functional responses of aortic rings from 11 $\beta$ HSD1<sup>-/-</sup> mice bathed in culture medium.**

<b>11<math>\beta</math>HSD1<sup>-/-</sup> (n)</b>			
	<b>Intact (6)</b>	<b>Denuded (6)</b>	<b>p-values</b>
<b>KPSS</b>			
<b>E<sub>max</sub>, mN/mm</b>	1.4 $\pm$ 0.2	0.56 $\pm$ 0.1	0.19
<b>5HT</b>			
<b>E<sub>max</sub>, mN/mm</b>	3.28 $\pm$ 0.6	1.41 $\pm$ 0.2	<b>0.02*</b>
<b>E<sub>max</sub>, %KPSS</b>	238.6 $\pm$ 22.5	295.9 $\pm$ 39.4	<b>0.01*</b>
<b>pD<sub>2</sub></b>	6.74 $\pm$ 0.1	6.53 $\pm$ 0.1	0.18
<b>NA</b>			
<b>E<sub>max</sub>, mN/mm</b>	1.59 $\pm$ 0.4	0.84 $\pm$ 0.2	0.09
<b>E<sub>max</sub>, %KPSS</b>	120.3 $\pm$ 9.4	175.7 $\pm$ 35.9	<b>0.01*</b>
<b>pD<sub>2</sub></b>	7.64 $\pm$ 0.3	7.13 $\pm$ 0.2	0.16
<b>ACh</b>			
<b>E<sub>max</sub>, %5HT</b>	1.59 $\pm$ 0.4	0.84 $\pm$ 0.2	0.09
<b>-LogIC<sub>50</sub></b>	7.8 $\pm$ 0.1	not calculated	
<b>SNP</b>			
<b>E<sub>max</sub>, %5HT</b>	73.6 $\pm$ 26.6	87.1 $\pm$ 30.7	<b>0.02*</b>
<b>-LogIC<sub>50</sub></b>	8.14 $\pm$ 0.2	7.69 $\pm$ 0.5	0.57

Maximal relaxation (%) and contraction (E<sub>max</sub>) responses and sensitivity (expressed as – logIC<sub>50</sub> or pD<sub>2</sub>) for 125mM K<sup>+</sup> (KPSS), 5-hydroxytryptamine (5HT), acetylcholine (ACh), sodium nitroprusside (SNP) and noradrenaline (NA). Responses were obtained using rings of thoracic aortae isolated from 11 $\beta$ HSD1<sup>-/-</sup> mice. Values for each concentration-response curve were produced in Dulbecco's modified essential media without L-arginine (DMEM-L-Arg buffer). Removal of endothelium produced an alteration in responses broadly similar to those observed with rings from C57Bl/6 mice. All data represent mean  $\pm$  SEM, n=6, compared by unpaired Student's t-test, \*indicates a significant difference between intact and denuded arteries, p<0.05.

#### **6.4.2. Effect Of 7-Oxysterols On Vascular Function *In Vitro***

The purpose of these experiments was to establish the effect of 7-oxysterols on functional response of mouse aortae *ex vivo*. The effects of 7-KC (25  $\mu$ M) or 7 $\beta$ OHC (20  $\mu$ M) on the function of mouse thoracic aorta were monitored after short (4h) and prolonged (overnight) incubation.

##### **6.4.2.1. Short-Term Exposure Of Aortic Rings to 7-Keto-cholesterol And 7 $\beta$ -Hydroxycholesterol**

Exposure of aortae from C57Bl/6 mice to high physiological concentrations of 7-oxysterols (25  $\mu$ M 7KC or 20  $\mu$ M 7 $\beta$ OHC), for 4h did not attenuate the maximal contractile response to high concentration potassium solution (KPSS, during the standard start procedure). Exposure of mouse aorta to 7-KC, but not 7 $\beta$ OHC produced a small reduction ( $p=0.049$ ) in NA-induced maximum contraction ( $E_{\max}$ ), but had no effect on the sensitivity ( $pD_2$ ), see Table 6 – 3 and Figure 6.4 – 2.

Pre-treatment of vessels with either 7-KC or 7 $\beta$ OHC did not alter the maximal relaxation responses or the sensitivity to the endothelium-dependent vasodilator ACh. There was a suggestion that the maximal response to endothelium-independent, SNP-mediated vasorelaxation was increased after incubation with either 7-KC ( $p=0.05$ ) or 7 $\beta$ OHC ( $p=0.08$ ) but this did not achieve significance.



Table 6-3 Effect of 7-ketocholesterol (7-KC) and 7 $\beta$ -hydroxycholesterol (7 $\beta$ OHC) on relaxation and contraction of mouse aorta – short incubation.

**A) 7-KC**

<i>i) Emax</i>			
	Pre-treatment	Vehicle	7-Ketocholesterol
<b>KPSS</b>	1.9 $\pm$ 0.37	not calculated	not calculated
<b>5HT</b>	2.3 $\pm$ 0.30	3.01 $\pm$ 0.37	2.47 $\pm$ 0.26
<b>NA</b>	1.4 $\pm$ 0.24	0.79 $\pm$ 0.28	0.40 $\pm$ 0.08 *
<b>ACh</b>	not calculated	46.26 $\pm$ 5.32	47.13 $\pm$ 7.03
<b>SNP</b>	not calculated	104.8 $\pm$ 3.84	122.0 $\pm$ 8.31 *

*ii) Sensitivity*

	Pre-treatment	Vehicle	7-Ketocholesterol
<b>KPSS</b>	18.5 $\pm$ 1.51	not calculated	not calculated
<b>5HT</b>	6.8 $\pm$ 0.15	6.36 $\pm$ 0.08	6.55 $\pm$ 0.06
<b>NA</b>	7.7 $\pm$ 0.15	6.70 $\pm$ 0.34	7.92 $\pm$ 0.48
<b>ACh</b>	not calculated	6.32 $\pm$ 0.09	6.48 $\pm$ 0.09
<b>SNP</b>	not calculated	7.53 $\pm$ 0.22	7.56 $\pm$ 0.12

**B) 7 $\beta$ OHC**

<i>i) Emax</i>			
	Pre-treatment	Vehicle	7 $\beta$ -Hydroxycholesterol
<b>KPSS</b>	2.0 $\pm$ 0.22	not calculated	not calculated
<b>5HT</b>	2.3 $\pm$ 0.25	2.8 $\pm$ 0.28	3.3 $\pm$ 0.32
<b>NA</b>	1.1 $\pm$ 0.04	1.6 $\pm$ 0.36	1.8 $\pm$ 0.61
<b>ACh</b>	not calculated	47.7 $\pm$ 6.22	59.1 $\pm$ 4.78
<b>SNP</b>	not calculated	116.7 $\pm$ 10.3	121.7 $\pm$ 12.01 <sup>t</sup>

*ii) Sensitivity*

	Pre-treatment	Vehicle	7 $\beta$ -Hydroxycholesterol
<b>KPSS</b>	19.5 $\pm$ 2.51	not calculated	not calculated
<b>5HT</b>	6.6 $\pm$ 0.07	6.4 $\pm$ 0.04	6.5 $\pm$ 0.05
<b>NA</b>	6.9 $\pm$ 0.25	6.9 $\pm$ 0.17	6.8 $\pm$ 0.22
<b>ACh</b>	not calculated	7.3 $\pm$ 0.32	7.1 $\pm$ 0.16
<b>SNP</b>	not calculated	7.9 $\pm$ 0.11	8.2 $\pm$ 0.11

**(A)** Responses of endothelium-intact aortic rings to 7-ketocholesterol (7-KC, 25  $\mu$ M) or vehicle (ethanol containing 50  $\mu$ g/ml butylated hydroxytoluene, BHT): **i)** Maximal relaxation ( $E_{\max}$  %5HT) and contraction (mN/mm), **ii)** Sensitivity (expressed as  $-\text{LogIC}_{50}$  for relaxants or  $\text{pD}_2$  for vasoconstrictors). **(B)** Responses of endothelium-intact aortic rings to 7 $\beta$ -hydroxycholesterol (7 $\beta$ OHC, 20  $\mu$ M) and vehicle (ethanol, containing 50  $\mu$ g/ml BHT): **i)** Maximal relaxation ( $E_{\max}$  %5HT) and contraction (mN/mm), **ii)** Sensitivity (expressed as  $-\text{LogIC}_{50}$  or  $\text{pD}_2$ ). Comparisons were made between vehicle-treated and 7-KC-treated rings, and between vehicle-treated and 7 $\beta$ OHC-treated rings for responses to each drug. 7-KC, but not 7 $\beta$ OHC produced a small reduction ( $p=0.049$ ) in Na-induced maximum contraction, but had no effect on the sensitivity. All values represent mean  $\pm$  SEM, compared by unpaired Student's t-test (vehicle vs. 7-KC, vehicle vs. 7 $\beta$ OHC),  $n=6-8$ , \* $p<0.05$ , <sup>t</sup> trend. NA, noradrenaline; 5HT, 5-hydroxytryptamine; ACh, acetylcholine; SNP, sodium nitroprusside.

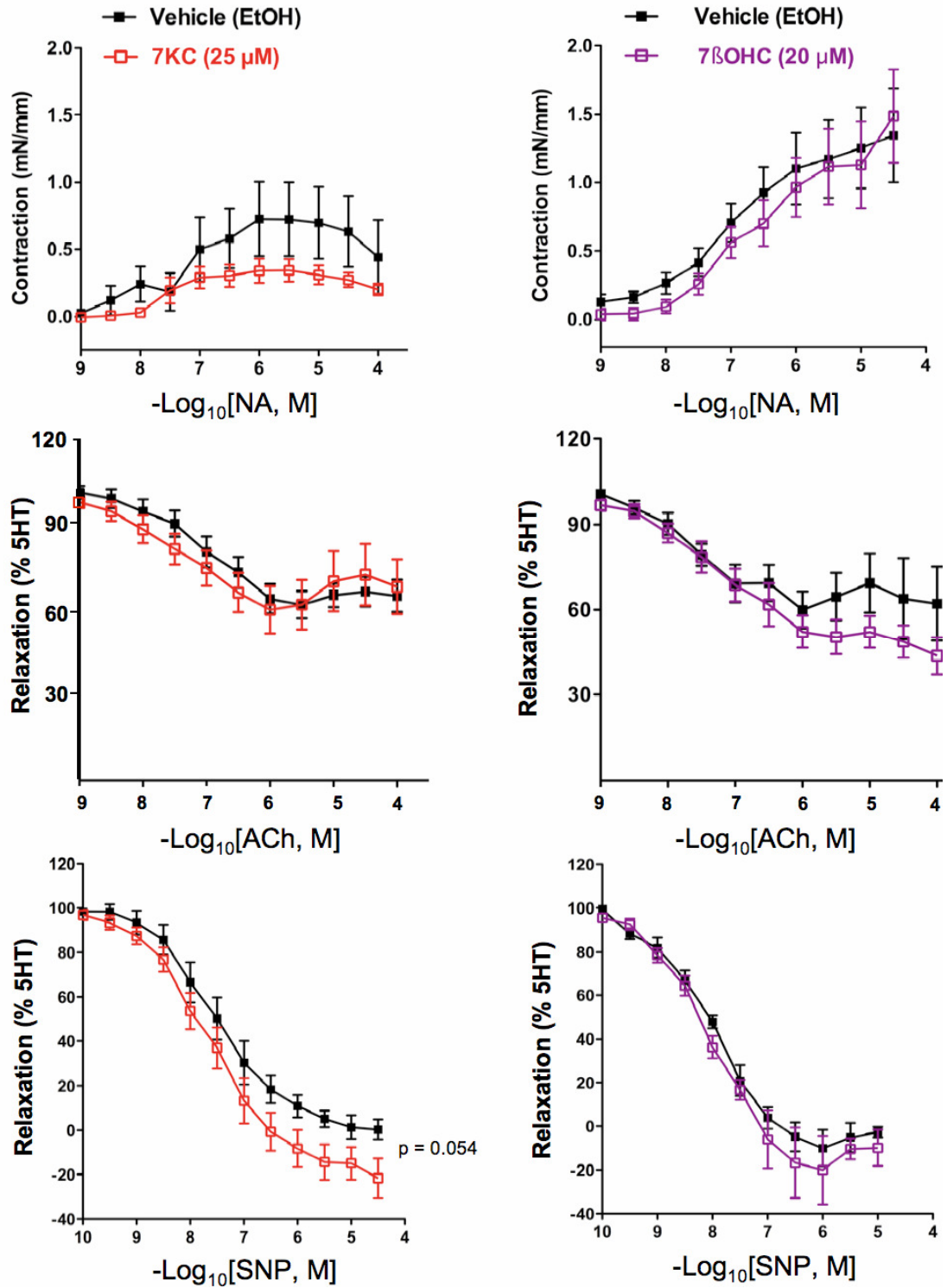


Figure 6.4-2 Influence of 7-oxysterols on vascular reactivity of mouse aorta – short incubation.

Aortae from C57Bl/6 mice were incubated (4 hours) with 7-KC (25  $\mu$ M, red), 7 $\beta$ OHC (20  $\mu$ M, purple) or vehicle (black squares). Responses to noradrenaline (NA), acetylcholine (ACh) and sodium nitroprusside (SNP) were tested in endothelium-intact vessels. Relaxations were expressed on a scale where the response to 5HT represented 100% and return to baseline was expressed as 0%. Incubation with 7-KC, but not 7 $\beta$ OHC, produced a small reduction of NA-mediated contraction ( $p=0.04$ ). Incubations had no effect on ACh-mediated relaxation but 7-KC produced a trend towards increased SNP-mediated relaxation ( $p=0.054$ ). All points represent mean  $\pm$  SEM, compared by 1-way ANOVA with Tukey's post hoc test,  $n=6-8$ .

#### **6.4.2.2. Long-Term Exposure Of Aortic Rings to 7-Keto-cholesterol And 7 $\beta$ -Hydroxycholesterol**

Exposure of aortae from C57Bl/6 mice to 7-KC (25  $\mu$ M) or 7 $\beta$ OHC (20  $\mu$ M) for 24 h did not attenuate the maximum contractile response to potassium solution (KPSS, during the start of general procedure) (Table 6 - 4). Furthermore, prolonged incubation with 7-oxysterols did not alter aortic contraction in response to 5HT or KPSS (Table 6 - 4); Figure 6.4 – 3).

Exposure of mouse aorta to 7-KC produced a trend ( $p=0.05$ ) for reduction of maximum contraction ( $E_{max}$ ) compared with vehicle control, whereas sensitivity ( $pD_2$ ) remained unchanged. There was not a significant difference observed between contractile responses of aortic rings to NA, after prolonged treatment with 7 $\beta$ OHC/vehicle (Table 6 - 4, Figure 6.4 – 3).

Prolonged treatment of aortic rings with either 7-oxysterol did not alter maximal relaxation responses to the endothelium-dependent vasodilator ACh. Similarly, there was no difference between the relaxation responses to the endothelium-independent vasodilator SNP after prolonged incubation of aortic rings with either 7-KC or 7 $\beta$ OHC (Table 6 - 4, Figure 6.4 – 3).

Table 6-4 Effect of 7-ketocholesterol and 7 $\beta$ -hydroxycholesterol on relaxation and contraction of mouse aorta – long incubation.

**A) 7-KC**

**i) *E<sub>max</sub>***

	Vehicle	7-Ketocholesterol	p - value
<b>KPSS</b>	3.03 $\pm$ 0.14	2.84 $\pm$ 0.22	0.39
<b>5HT</b>	4.03 $\pm$ 0.24	3.63 $\pm$ 0.33	0.21
<b>ACh</b>	66.9 $\pm$ 4.48	75.4 $\pm$ 6.42	0.46
<b>NA</b>	2.28 $\pm$ 0.34	1.56 $\pm$ 0.48	<b>0.05 *</b>
<b>SNP</b>	108.6 $\pm$ 4.96	106 $\pm$ 1.54	0.57

**ii) *Sensitivity***

	Vehicle	7-Ketocholesterol	p - value
<b>KPSS</b>	29.4 $\pm$ 1.14	38.4 $\pm$ 3.20	0.49
<b>5HT</b>	6.61 $\pm$ 0.05	6.60 $\pm$ 0.12	0.88
<b>ACh</b>	7.47 $\pm$ 0.13	7.35 $\pm$ 0.21	0.90
<b>NA</b>	7.79 $\pm$ 0.16	7.94 $\pm$ 0.33	0.50
<b>SNP</b>	7.04 $\pm$ 0.17	6.95 $\pm$ 0.19	0.66

**B) 7 $\beta$ OHCH**

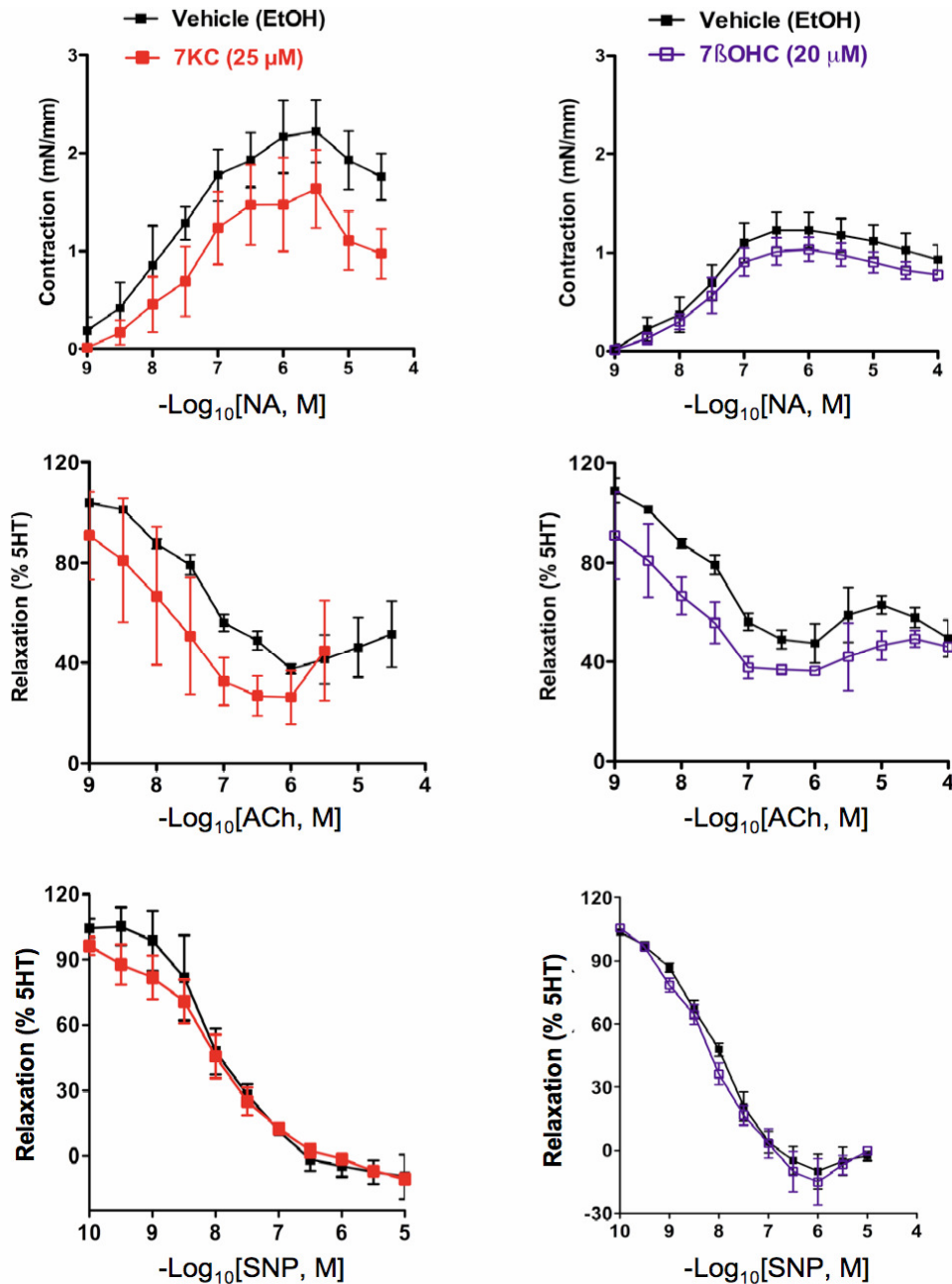
**i) *E<sub>max</sub>***

	Vehicle	7 $\beta$ -Hydroxycholesterol	p - value
<b>KPSS</b>	2.98 $\pm$ 0.46	2.79 $\pm$ 0.32	0.29
<b>5HT</b>	2.43 $\pm$ 0.14	2.88 $\pm$ 0.15	0.23
<b>ACh</b>	46.4 $\pm$ 5.56	60.3 $\pm$ 5.15	0.86
<b>NA</b>	1.21 $\pm$ 0.14	1.16 $\pm$ 0.12	0.25
<b>SNP</b>	104.6 $\pm$ 3.46	109.5 $\pm$ 1.94	0.47

**ii) *Sensitivity***

	Vehicle	7 $\beta$ -Hydroxycholesterol	p - value
<b>KPSS</b>	39.2 $\pm$ 0.24	36.4 $\pm$ 1.22	0.48
<b>5HT</b>	6.51 $\pm$ 0.15	6.90 $\pm$ 0.32	0.79
<b>ACh</b>	7.17 $\pm$ 0.15	6.97 $\pm$ 0.31	0.45
<b>NA</b>	6.55 $\pm$ 0.06	6.24 $\pm$ 0.03	0.50
<b>SNP</b>	6.84 $\pm$ 0.16	6.72 $\pm$ 0.19	0.26

Responses of endothelium-intact aortic rings were measured following 24h incubation with **(A)** 7-ketocholesterol (7-KC, 25  $\mu$ M) or **(B)** 7 $\beta$ -hydroxycholesterol (7 $\beta$ OHC, 20  $\mu$ M) and compared with vehicle (ethanol containing 50  $\mu$ g/ml butylated hydroxytoluene, BHT)-treated controls: *i*) Maximal relaxation ( $E_{\max}$  %5HT) and contraction (mN/mm), *ii*) Sensitivity (expressed as  $-\log IC_{50}$  for relaxants or  $pD_2$  for vasoconstrictors). All values represent mean  $\pm$  SEM, compared by unpaired Student's t-test (vehicle vs. 7-KC; vehicle vs. 7 $\beta$ OHC),  $n=4-6$ , \* $p<0.05$ . NA, noradrenaline; 5HT, 5-hydroxytryptamine; ACh, acetylcholine; SNP, sodium nitroprusside.



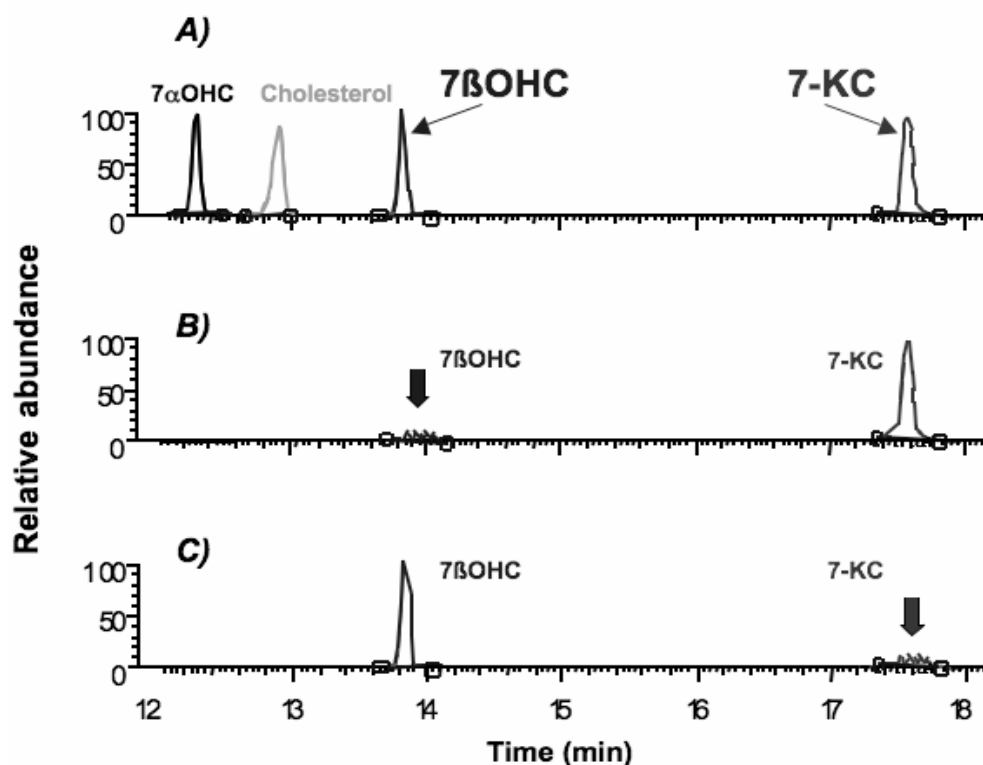
**Figure 6.4-3 Influence of 7-oxysterols on vascular reactivity of mouse aorta – long incubation.**

Endothelium-intact aortae from C57Bl/6 mice ( $n=4$ ) were incubated (24h) with 7-KC (25  $\mu\text{M}$ , red), 7 $\beta$ OHC (20  $\mu\text{M}$ , purple) or vehicle (black squares). Responses to noradrenaline (NA), acetylcholine (ACh) and sodium nitroprusside (SNP) were tested. Relaxations were expressed on a scale where the response to 5HT represented 100% and return to baseline was expressed as 0%. Incubation of aortic rings with 7-KC, but not 7 $\beta$ OHC, produced a trend for reduction of NA-mediated contractions ( $p=0.05$ ) but neither oxysterol had any effect on ACh-mediated or SNP-mediated relaxations compared with vehicle control. All points represent mean  $\pm$  SEM, compared by 1-way ANOVA with Tukey's post hoc test,  $n=4-6$ .

#### **6.4.2.3. Are 7-Oxysterols Metabolised by 11 $\beta$ HSD1 in the Aortic Wall?**

Of particular interest for this thesis was metabolism of 7-oxysterols by 11 $\beta$ HSD1 in the vessel wall. Formation of products from both 7-KC and 7 $\beta$ OHC was monitored by GC/MS after the short and long incubations with 7-oxysterols (Figure 6.4 – 4 A). The individual substrates were successfully recovered from the medium after both incubations as shown by single peaks of 7-KC or 7 $\beta$ OHC on the GC/MS chromatograms (Figure 6.4 – 4 B and C). After short (4h) incubation, 7-KC was not detected in medium from vessels exposed to 7 $\beta$ OHC. Similarly, 7 $\beta$ OHC was not detected in medium from vessels exposed to 7-KC. After prolonged (24h) exposure, 7 $\beta$ OHC was detected in two (of eight) incubations with 7-KC whilst 7-KC was detected in two (of six) incubations with 7 $\beta$ OHC.





**Figure 6.4-4 GC/MS chromatogram of derivatised 7-oxysterols detected in the medium upon incubation of vessels.**

A) Separation by GC/MS of derivatised standards: 7 $\alpha$ -hydroxycholesterol (7 $\alpha$ OHC); 7 $\beta$ -hydroxycholesterol (7 $\beta$ OHC); cholesterol and 7-ketocholesterol (7-KC). Metabolism of 7-oxysterols by B) reduction (7-KC $\rightarrow$ 7 $\beta$ OHC) and C) dehydrogenation (7 $\beta$ OHC $\rightarrow$ 7-KC) of substrates (20 - 25  $\mu$ M) by 11 $\beta$ HSD1 were assessed in the aortic rings (1 - 2 mm) from C57Bl/6 mice following a long (24 h) incubation. Only substrates (7-KC and 7 $\beta$ OHC, respectively) were detected in each reaction, suggesting that conversion to respective products (7 $\beta$ OHC and 7-KC) did not occur. GC/MS, Gas chromatography/Mass spectrometry.

## 6.5. Discussion

The work presented in this chapter tested the hypothesis that metabolism of 7-oxysterols by 11 $\beta$ HSD1 in the vascular wall may impair functional responses of murine aortae *ex vivo*. The initial aim was to determine whether 7-oxysterols alter vascular function in aortic rings from C57Bl/6 mice. In order to achieve this it was necessary to develop a method to allow sustained incubation, and functional assessment, in arterial sections bathed in culture medium, rather than PSS. It was demonstrated that performing functional investigations in L-arginine deplete DMEM did not dramatically alter responses to vasoconstrictor or vasodilators. Subsequent experiments also confirmed that the impact of endothelial cell removal could also be demonstrated in aortic rings from 11HSD1 knockout mice, using DMEM-L-Arg rather than PSS. Using this approach, it was demonstrated that exposure to 7-KC or 7- $\beta$ OHC for up to 24 hours did not alter functional responses of the mouse aorta. Furthermore, analysis of culture medium found very little evidence of metabolism of these 7-oxysterols by aortic 11 $\beta$ HSD1 *ex vivo*.

### 6.5.1. Use of culture medium for functional investigations and prolonged incubation with 7-oxysterols

In order to assess the impact of exposure to 7-oxysterols on vascular function, it was desirable to perform extended incubations of aortic rings with these compounds; particularly given the potential for oxysterols to interaction with nuclear hormone receptors. This was not possible if PSS was used as the incubating medium as this only maintains arterial function for several hours (Christy, 2003). Previous work has demonstrated that arterial function can be maintained for up to ~24hours if vessels are incubated in DMEM (Christy *et al.*, 2003)). It was valuable, therefore, to determine whether the actual functional analyses could also be performed in culture medium (rather than PSS). This approach has been used successfully by others (Brem *et al.*, 1997; Christy *et al.*, 2003). Initial difficulties encountered with excessive foaming from bovine serum albumin (BSA) in the myograph were

countered by using charcoal-stripped FBS (which also ensured that no steroids were present in the culture medium). The absence of L-arginine was important for these experiments, being that L-arginine is a precursor for the synthesis of NO by nitric oxide (NO) synthase, therefore use of DMEM without L-arginine assured no exogenous supply of substrate for the production of NO by the aortic rings *in vitro*. Indeed, previous work by Christy *et al.*, (2003) has demonstrated that DMEM containing high concentrations of L-arginine ameliorated the functional abnormalities associated with endothelial cell dysfunction in 11 $\beta$ HSD2 deficient mice. Nevertheless, the use of DMEM deprived of L-arginine has allowed to maintain the viability and vascular function of aortic rings. Overall, performing myographic investigations in arteries bathed in DMEM-L-Arg produced responses similar to those obtained in PSS. Responses to vasoconstrictors were initially slower to develop in DMEM-L-Arg, but the maximal responses achieved were very similar to those in PSS and were therefore predicted to be the same in normal DMEM. The demonstration that vasoconstrictor and (endothelium-dependent and -independent) vasorelaxant responses were similar whether obtained in DMEM-L-Arg or PSS, confirmed that myographic experiments could be performed in culture medium. This was important as it allowed continued exposure to 7-oxysterols during functional investigations.

In addition to assessing the affect of 7-oxysterols on function of aorta isolated from control (C57Bl/6), it was originally intended that similar experiments would be performed in vessels from 11 $\beta$ HSD1 knockout mice. This would clarify the influence of aortic 11 $\beta$ HSD1 activity on modulating the functional effects of the 7-oxysterols. It was necessary, therefore, to confirm that functional responses could be obtained when aortae isolated from mice of both genotypes were bathed in DMEM-L-Arg. The data obtained demonstrated that removal of the endothelium abolished ACh-mediated relaxation and enhanced the maximum response to SNP. This is consistent with the effect of removing a fully functional endothelium (Hadoke *et al.*, 2001). Furthermore, endothelial denudation provoked enhanced contraction to 5HT and, particularly, to NA; consistent with the observation that denudation removes the physiological antagonism of contraction produced by basal and stimulated release of

NO from the endothelium (Bullock *et al.*, 1986; Martin *et al.*, 1986). If anything, the effects of endothelial cell removal were more apparent in aortae from 11 $\beta$ HSD1 knockout mice than in C57Bl/6; perhaps due to the greater number of vessels used and improved myographic technique. The data obtained in aortae from 11 $\beta$ HSD1 knockout mice suggest normal contractile and relaxant function in these animals. This is consistent with data obtained from similar knockout mice on an MF1 background (Hadoke *et al.*, 2001). It is an important observation as pharmacological inhibition of 11 $\beta$ HSD1 dehydrogenase activity has been associated with an enhanced response to vasoconstrictors, whereas inhibition of the reductase reaction produces attenuated constriction (Morris *et al.*, 2003).

### **6.5.2. Functional Responses Of Mouse Thoracic Aorta To 7-Oxysterols**

Hyperlipidaemia and atherosclerosis are associated with abnormalities of vascular function (Crauwels *et al.*, 2003; Jiang *et al.*, 2001). Components of an atherosclerotic plaque, such as oxidized low-density lipoproteins (oxLDL), have cytotoxic properties and may impair vascular function. A large part of oxidized LDL (40%) contains 7-oxysterols, ~30% of which is 7-KC. There is a good body of work showing that accumulation of oxysterols into endothelial and aortic smooth muscle cells leads to apoptotic cell death (Rho *et al.*, 2005). Further deposition of oxidized cholesterol and cholesterol oxidation products (such as 7-oxysterols) may contribute to vascular toxicity (Vine *et al.*, 1998). Such a high-lipid environment may not only impair vascular function but influence the stability of atherosclerotic plaque in human and mice (Gaut and Heinecke, 2001; Libby, 2002a). Furthermore increased levels of oxidized-LDL in atherosclerosis and hyperlipidamia are known cause of foam-cell formation. The build-up of these hormones in the vessel may also influence contractility and thus alter vascular tone and function as explored here.

The *ex vivo* culture of aortic rings, as used for the myography studies, has not previously been applied to assess inter-conversion of 7-oxysterols. This approach was able to demonstrate that it is possible to preserve stability of 7-oxysterols upon

incubation, as both 7-KC and 7 $\beta$ OHC were successfully recovered from DMEM. It had been postulated that 7-oxysterols may be taken up by the vessels during incubation but the recovery of 7-oxysterols indicates that this did not happen. Under conditions used, the measurement of 11 $\beta$ HSD1 activity was not always achieved during the incubation of aortic rings, possibly due to the small amount of tissue used. It was subsequently established that the use of whole aortae was needed to obtain measurable conversion of 7-oxysterols by 11 $\beta$ HSD1 as reported in Chapter 3.

Despite previous reports detailing adverse effects of exposure of vascular rings to 7-oxysterols *ex vivo* (Deckert *et al.*, 1998), preparing solutions in the concentration range described in the literature proved surprisingly difficult (as the oxysterols came out of solution at high concentrations). The approach ultimately adopted was involved preparing 7-oxysterol solutions in DMEM containing FCS (Built, H. *et al.*, personal communication). In addition, an antioxidant (BHT) was included in the solution to prevent oxidative degradation of the lipid during prolonged incubation (as described by Deckert *et al.*, 1998). BHT is not known to affect NO production and endothelium-dependent relaxation *ex vivo*. Indeed, BHT did not alter histamine-induced NO production in cultured HUVECs (Deckert *et al.*, 2002).

Unexpectedly, given published experience with these compounds, incubation of murine aortae with 7-oxysterols did not cause major functional impairment. The small reduction in NA-mediated contractions after short-term incubation with 7-KC is unlikely to be due to changes in endothelial cell function as responses to ACh and SNP were unaltered. Furthermore, the lack of effect on 5HT and KPSS-mediated contraction suggest that this alteration was specific to the  $\alpha$ -adrenoceptor-mediated responses. The mechanism underlying this change, and the reason why it was not evident after 24h incubation, are unclear. Deckert *et al.* previously aimed to dissect out the mechanism of 7-KC action on vascular function, demonstrating that 7-KC inhibits endothelium-dependent relaxations to ACh (Deckert *et al.*, 1998; Deckert *et al.*, 2002; Deckert *et al.*, 1997). The current studies performed using murine aortae have produced conflicting findings to those obtained by Deckert and colleagues in rabbit aorta. This may be due to species differences (although this seems unlikely)

but several concerns became evident during the work in mice. As stated (above), it was difficult to achieve the high concentrations of oxysterols (50  $\mu\text{M}$ ) achieved in these previous studies, without sample precipitation. Hence the experiments reported here used lower (although physiologically high) concentrations of 7-oxysterols (20 – 25  $\mu\text{M}$ ). It is also possible that the use of DMEM-L-Arg, rather than the PSS buffer used by Deckert *et al.*, (1998) accounts for the different results. It is possible that the use of DMEM without L-arginine has depleted the substrate for the endogenous NO production after an overnight incubation of the vessels. If the effects of 7-oxysterols have been on NO synthesis, then those might have been masked by the given experimental conditions. Alternatively, the use of nitric oxide synthase inhibitor (L-NAME) may be considered to investigate the NO status of the vessels after an overnight incubation in DMEM. Indeed, these experiments might have been conducted if the changes in function were seen in response to either of 7-oxysterols when incubating the vessels in DMEM without L-arginine or PSS. Therefore the endothelial dysfunction seen in the previous work by Deckert *et al.*, may have been (at least partly) the result of extended incubation in PSS, although why this was differentially affected by the presence of 7-oxysterols is unclear.

One other study, published during the course of this thesis, demonstrated a lack of inhibition of endothelium-dependent relaxation to acetylcholine either after incubation of murine aortic rings with physiologically high levels of 7-KC (205  $\mu\text{M}$ ), or after subcutaneous treatment of mice with 7-KC (10 mg/kg/day, 1 week) (Kiss *et al.*, 2006). *In vitro*, 7-KC induced oxidative stress in endothelial cells and further activated nuclear poly(ADP-ribose) polymerase (PARP) but these effects were attenuated upon inhibition of NOS activity. Thus 7-KC may impair NO-production in vascular endothelium and impair dimerisation of eNOS (Terasaka *et al.*, 2008). Adding to the plethora of published work, it is established that 7-KC, like oxLDL induces oxidative stress via formation of  $\text{O}_2^-$  in the vascular endothelium, which inactivates NO and forms peroxynitrate ( $\text{ONOO}^-$ ), thus consequently leading to oxidation of eNOS (Deckert, 1999; Fransen *et al.*, 2008; Leonarduzzi *et al.*, 2004; Lizard *et al.*, 2006).

Given the cytotoxic properties of oxysterols (Hughes *et al.*, 1994), their effects may be exerted through various other signalling events, influencing molecular interactions, like hedgehog signalling (Hh) pathway, (Christensen and Ott, 2007; King *et al.*, 2008). Moreover, the ability of 7-KC/7 $\beta$ OHC to induce apoptosis (by activating Fas ligand (FasL) to interact with its receptor (FasR)) is now being elucidated (Lordan *et al.*, 2008). It remains possible that formation of these species, their accumulation within the vessel wall and pro-apoptotic actions on vascular cells over a prolonged period of time may indeed impair endothelial function and eNOS activity. Alternatively, accumulation of 7-KC or 7 $\beta$ OHC (at high nM concentrations) may influence GC metabolism (see chapter 4). Therefore the main consequence of accumulation of 7-oxysterols may be perhaps to influence 11 $\beta$ HSD1-mediated glucocorticoid metabolism and the effects of glucocorticoids on the vasculature.

## **Chapter 7. General Discussion And Future Work**



## 7.1. General Overview

This thesis explored the role of 11 $\beta$ HSD1 in regulating the concentrations and actions of 7-oxysterols. The latter substrates are recognised as alternative substrates to glucocorticoids for metabolism by 11 $\beta$ HSD1. This work was prompted by the increasing interest in 11 $\beta$ HSD1 as a therapeutic target in atherosclerosis, a disease associated with an increased circulating and tissue burden of these oxidised lipids (Prunet *et al.*, 2006). To date there is a lack of understanding of how inhibition of 11 $\beta$ HSD1 can reduce plaque size and lipid accumulation (Hermanowski-Vosatka *et al.*, 2005; Iqbal *et al.*, 2009; Nuotio-Antar *et al.*, 2007), although mechanisms to reduce cardiovascular risk factors (e.g. insulin resistance, weight gain, angiogenesis (Small *et al.*, 2005; Wamil and Seckl, 2007)) may easily be explained by suppression of the actions of glucocorticoids.

Oxysterols (of which 7-oxysterols are a significant proportion Dzeletovic *et al.*, 1995) increase in abundance in hyperlipidaemia (Arca *et al.*, 2007; Vaya *et al.*, 2001) and their concentrations have been suggested as biomarkers of oxidative stress (Ferderbar *et al.*, 2007; Larsson *et al.*, 2007) and indices of susceptibility to atherosclerosis and cardiovascular disease (Alkazemi *et al.*, 2008). They are formed from cholesterol by various routes (Antonchick, 2007) but whether measurement of concentrations of oxysterols, as opposed to just those of cholesterol, to assess cardiovascular risk will provide added value is unknown. The main barrier to understanding their diagnostic potential is the lack of a defined molecular target. More recently 27-hydroxycholesterol has been demonstrated as a ligand for the estrogen receptor- $\beta$  (ER $\beta$ ) (DuSell *et al.*, 2008; Umetani *et al.*, 2007), but as yet a receptor has not been defined for 7-oxysterols (the possibility of activation of LXR has been investigated (Janowski *et al.*, 1999; Wang *et al.*, 2008) but largely dismissed (Olkkonen, 2008; Wamil *et al.*, 2008)). Nonetheless, it is clear that these compounds are readily subject to oxidation and may form free radicals in tissues and, therefore, if the burden of 7-oxysterols is increased within tissues (such as macrophages, plaques and vessels) oxidative damage may ensue. The question, then, may be posed as to whether the distinct 7-oxysterols (i.e. 7-KC, 7 $\beta$ OHC, 7 $\alpha$ OHC)

differ in their toxic actions. If this were true then the proportions of these compounds may influence disease progression and, hence, an understanding of factors controlling this balance (including 11 $\beta$ HSD1) would be of value.

Previous literature proposes a number of mechanisms of actions of 7-oxysterols in tissues, including: free radical production (Chisolm and Steinberg, 2000; Lukyanenko and Lukyanenko, 2009); altered vascular relaxation and contractility (Deckert *et al.*, 2002; Kiss *et al.*, 2006); induction of apoptosis (Li *et al.*, 2001; Vejux *et al.*, 2008). However, the actions of the distinct 7-oxysterols have not been explored systematically and the potential role of 11 $\beta$ HSD1 has been largely ignored. In this thesis, 7-oxysterols were not able to alter vascular contractility significantly, although a weak effect to reduce noradrenaline-mediated contraction was observed. Preliminary data (not presented) also implied that these agents do not promote apoptosis or induce free radical generation; therefore arguing against a direct molecular target, at least in vessels. If 7-KC and 7 $\beta$ OHC had produced different effects in the systems tested, one might have inferred a role for 11 $\beta$ HSD1 in gating these actions, and this may still be the case in other tissues where oxysterols accumulate and 11 $\beta$ HSD1 is expressed (e.g. macrophages, foam cells (Gilmour and *et al.*, 2006)); or via mechanisms as yet undefined. Whether, subsequent actions of 7-oxysterols on glucocorticoid production manipulate maintenance of normal endothelial function and atherosclerotic plaque stabilisation (Libby, 2002b), is a separate research question, which may follow beyond this thesis. Also, the question that remains is “does 11 $\beta$ HSD1 in the macrophages contribute to a local clearance of 7-KC?”. In spite of a lack of clear differences the actions of 7-KC versus 7 $\beta$ OHC, a beneficial effect of disruption of 11 $\beta$ HSD1 may lie in a suppression of total 7-oxysterol concentrations in tissues (as in the reduction of 7-oxysterols in hepatic microsomes from 11 $\beta$ HSD1<sup>-/-</sup> mice), thereby reducing the overall propensity to oxidative stress. Indeed transgenic disruption of 11 $\beta$ HSD1 substantially reduced the total amount of 7-oxysterols in tissues (as well as affecting the proportions).

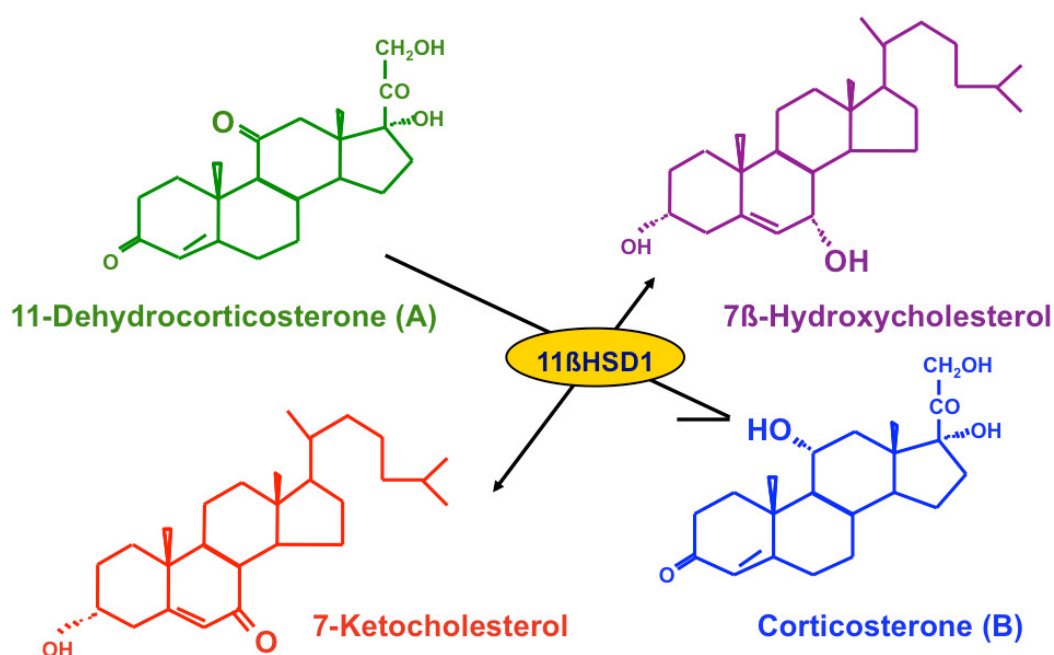
Metabolism of 7-oxysterols by murine 11 $\beta$ HSD1 could be easily demonstrated *in vitro*, in cultured cells and in *ex vivo* explants. The *in vitro* data presented here

confirmed reports in other species that 7-oxysterols were metabolised by 11 $\beta$ HSD1. However, some interesting differences were identified. Firstly, 7-oxysterols were metabolised at a slower and less efficient rate than glucocorticoids, rendering them poorer substrates for the enzyme. Secondly, the equilibrium of the inter-conversion between 7 $\beta$ OHC and 7-KC differed from that of glucocorticoids, in that the balance of the reaction favoured dehydrogenation of oxysterols, even in the presence of excess H6PDH. This contrasted with the characteristics of the reaction catalysed using glucocorticoids as substrates, in which reduction dominates (Bujalska *et al.*, 2005; Hewitt *et al.*, 2005; Lavery *et al.*, 2006). The cause of this distinction was not clear as *in silico* modelling suggested favourable binding of both 7 $\beta$ OHC and 7-KC for the active site of 11 $\beta$ HSD1. The difference may relate to transport and availability of 7-oxysterols within different subcellular compartments.

Experiments performed using *in vitro* cell culture suggested that cells preferentially excluded 7-KC, although both 7 $\beta$ OHC and 7-KC could penetrate the cell membrane. Little is known about active transport of 7-oxysterols within cells, although it is well recognised that membrane transporters exist for other species of oxysterol (Babiker and Diczfalusy, 1998; Baldan *et al.*, 2009). Thus it remains possible that 7-KC was unable to efficiently access the lumen of ER, where the active site of 11 $\beta$ HSD1 is located (Frick *et al.*, 2004; Odermatt *et al.*, 1999), in the models used. Assessment of metabolism within primary hepatocytes, which retain more comprehensive characteristics of the intact liver, may provide further insight into 11 $\beta$ HSD1 substrate preference. In mice with transgenic disruption of 11 $\beta$ HSD1, at least in hepatic microsomes, the balance of 7-oxysterols was changed in favour of 7 $\beta$ OHC, corroborating the predominant dehydrogenation activity. This difference was not, however, evident in plasma where an increase in 7-KC was observed upon disruption of 11 $\beta$ HSD1, potentially contributing towards storage of 7-KC in the vasculature for metabolism via other more dominant routes (Jessup *et al.*, 2005). However, the difference between levels of 7-oxysterols in tissues *vs.* plasma may reflect the fact that circulating pools of esters of 7-oxysterols (Freeman *et al.*, 2005) were not quantified.

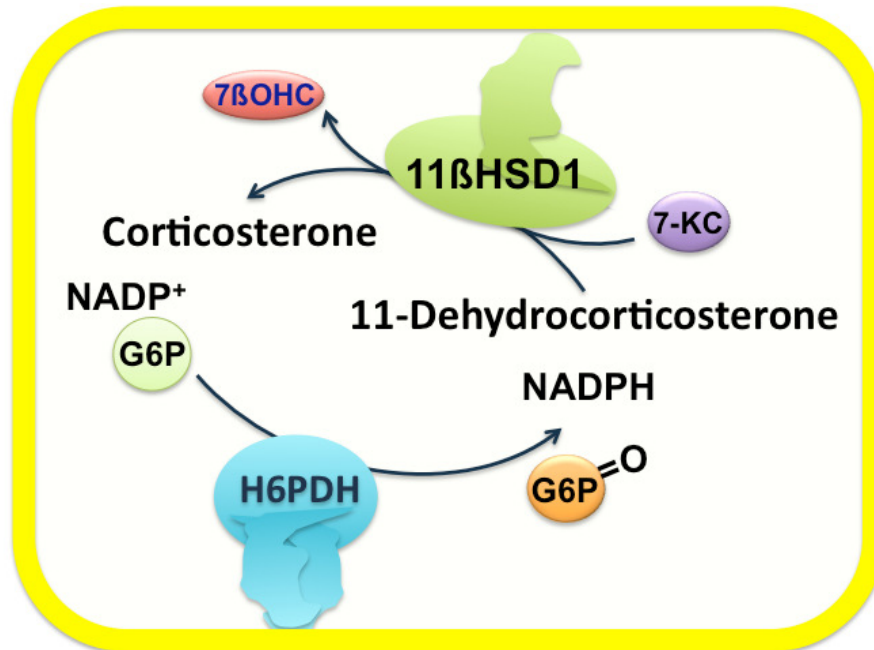
Whilst the change in balance in 7-oxysterols following 11 $\beta$ HSD1 inhibition may not influence physiological processes directly, an indirect role for 7-oxysterols was revealed. Competitive inhibition of metabolism of conventional glucocorticoid substrates by 7-oxysterols (Figure 7.1 – 1) was demonstrated, mainly for 7 $\beta$ OHC to prevent dehydrogenation of active glucocorticoid. Importantly this effect was limited to 7-oxysterols and to some extent to cholesterol, thus presenting them with a function distinct from other side-chain oxidised sterols. Therefore, the concentrations of 7-oxysterols may regulate the balance between active and inactive glucocorticoids, beyond the actions of H6PDH (Figure 7.1 – 2).

The concentrations of oxysterols found within the body are of central importance when considering the likelihood of their inhibitory effect on glucocorticoid metabolism *in vivo*. The  $K_i$  values of inhibition of GC metabolism were in a range of 1 - 8  $\mu$ M, and the circulating concentrations of 7-oxysterols (although they increase with age) are only in the nM range in normolipidaemia, but in dyslipidaemia may rise to  $\sim$ 100 $\mu$ M. Their concentrations under normal conditions demonstrate a circadian pattern (Noshiro *et al.*, 2007; Reilly *et al.*, 2007) but even then the maximum achieved in healthy individuals is unlikely to influence glucocorticoid actions. This suggests, therefore, that concentrations of 7-oxysterols may only influence the balance of active and inactive glucocorticoid (favouring production of the active species) during disease pathogenesis. Again, this effect will be most marked in tissues where oxysterols accumulate and 11 $\beta$ HSD1 is expressed. Indeed sites such as the liver, macrophages, foam cells and atherosclerotic plaques meet these criteria. These tissues may, therefore, be exposed to increased active glucocorticoids under atherosclerotic conditions, as well as suffering from increased oxidative stress induced by excess oxysterols. Excess glucocorticoid production, like that displayed in patients with Cushing's syndrome is also associated with dyslipidaemia and metabolic syndrome. The decreasing glucocorticoid action in specific tissues (such as adipose, liver and the vasculature) might protect against the



**Figure 7.1-1 The cross-talk between metabolism of glucocorticoids and 7-oxysterols by 11 $\beta$ HSD1.**

11 $\beta$ -Hydroxysteroid dehydrogenase type 1 (11 $\beta$ HSD1) catalyses the metabolism both of both 7-oxysterols and glucocorticoids. Conversion of 7-ketocholesterol (7-KC) to 7 $\beta$ -hydroxycholesterol (7 $\beta$ OHc). may occur alongside the metabolism of 11-dehydrocorticosterone (A) to corticosterone (B) in rodents. The two pathways compete for the metabolism by their mutual enzyme target.



**Figure 7.1-2 7-Oxysterols regulate the balance between active and inactive glucocorticoids, beyond the actions of H6PDH.**

11β-Hydroxysteroid dehydrogenase type 1 (11βHSD1) catalyses the metabolism of glucocorticoids (inactive 11-dehydrocorticosterone to active corticosterone) and 7-oxysterols (interconversion of 7-ketocholesterol (7-KC) and 7β-hydroxycholesterol (7βOHC)) in the cell. Hexose-6-phosphate dehydrogenase (H6PDH) converts glucose-phosphate (G6P) to 6-phospho gluconolactone (G6P=O) thus generating NADPH for the 11βHSD1 reactions within the endoplasmic reticulum (ER). 7-Oxysterols can compete with glucocorticoids for metabolism by 11βHSD1 and thereby regulate the balance between the active and inactive glucocorticoid.

detrimental metabolic consequences of obesity, diabetes and atherosclerosis (Hale and Wang, 2008).

It may, therefore, be postulated that inhibition of 11 $\beta$ HSD1 therapeutically will have the combined action of impairing activation of glucocorticoids directly but also in lowering the harmful actions, and inducing a change in the balance, of 7-oxysterols. Whether the ensuing increase in cellular and circulating 7 $\beta$ OHC will be sufficient to alter 11 $\beta$ HSD1 activity and thus influence the concentrations of active glucocorticoid will depend on the relative concentrations of these two classes of substrate.

The recent use of 11 $\beta$ HSD1 inhibitors for treatment of atherosclerosis (Hermanowski-Vosatka *et al.*, 2005) has extended the possible clinical application of these compounds. However, this potential role suffers from the fact that there have been no clinical trials with 11 $\beta$ HSD1 inhibitors in patients with cardiovascular disease. Furthermore, the mechanism through which these compounds reduce lesion size has not been identified. It is conceivable that the beneficial effects of 11 $\beta$ HSD1 inhibition occur through prevention of 7-oxysterol inter-conversion rather than (or as well as) glucocorticoid metabolism. To determine which of these is most important development of atherosclerotic plaques could be studied in 11 $\beta$ HSD1 knockout mice (e.g. ApoE<sup>-/-</sup>, 11 $\beta$ HSD1<sup>-/-</sup>, (Hadoke *et al.*, 2008)) following adrenalectomy (to remove endogenous glucocorticoids). In addition, the influence of selective 11 $\beta$ HSD1 inhibitors on 7-oxysterol metabolism could be ascertained in clinical trials if measurements of plasma oxysterols (in particular 7-KC and 7 $\beta$ OHC and calculation of the ratio of these two species) were performed (Courtney *et al.*, 2007)

In Chapter 5 of this thesis, a study was performed using an 11 $\beta$ HSD1 inhibitor (Compound 544) but in spite of reducing plaque size (Hermanowski-Vosatka *et al.*, 2005; Iqbal *et al.*, 2009), tissue concentrations of 7-oxysterols were not altered. Of interest, this compound also failed to inhibit 7-oxysterol metabolism *in vitro*, whereas it efficiently prevented reduction of glucocorticoids. This suggests that the presence of the drug in the active site of the enzyme is able to induce allosteric modifications in the binding of glucocorticoid but not of 7-oxysterols. The nature of

interactions between Compound 544 and the active site have not been modelled; such an approach might elucidate why conversion of these different substrates is not affected in the same way by the inhibitor. It is, as yet, hard to predict whether it will be beneficial to design the ability to inhibit 7-oxysterol metabolism into potential new 11 $\beta$ HSD1 inhibitors. Although, arguably, a total reduction in oxidised lipids due to lowering of the levels of cholesterol (as reported in chapter 5), would be advantageous. In any case it will be important to measure circulating glucocorticoids, 7-oxysterols and cholesterol when interpreting the effects and efficacy of these drugs. These measurements would have complemented the inhibitor study presented by Hermanowski-Vosatka *et al.*, (2005) and would have given a coherent mechanistic understanding into atheroprotective effect of inhibition of 11 $\beta$ HSD1. It is possible that upon inhibition of 11 $\beta$ HSD1 in the vascular cells, other cell types, such as macrophages, may take over the clearance of 7-KC. In particular, the 27-hydroxylase (CYP27A1) route (Brown *et al.*, 2000a) may compensate for the inhibition of 11 $\beta$ HSD1 by clearing 7-KC via formation of 27-hydroxyl-7-KC-cholesterol (Lee *et al.*, 2006).

In summary the work from this thesis has established that: **1)** murine 11 $\beta$ HSD1-mediates the metabolism of 7-oxysterols (7-KC and 7 $\beta$ OHC). **2)** These alternative substrates (7-oxysterols) compete with the glucocorticoids for metabolism by 11 $\beta$ HSD1 and, therefore, may control the production of active glucocorticoid (corticosterone) by the cell. **3)** Cellular cholesterol levels *in vitro* determine 7-oxysterol levels and, thus, may have further inhibitor actions on glucocorticoid metabolism by 11 $\beta$ HSD1. **4)** Genetic deletion (or inhibition) of 11 $\beta$ HSD1 in mice has confirmed that 11 $\beta$ HSD1 and cholesterol, regulate the plasma and tissue levels of 7-oxysterols. **5)** Individual 7-oxysterols do not cause impairment in endothelium-dependent vascular reactivity *in vitro*.



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